ATTACHMENTS TO SUPPLEMENTAL RESPONSE:

1. Hammer et al., "Production of transgenic rabbits; sheep and pigs by micro-injection, Nature, 315(6021):680-683 (1985);

2. Knight et al., "Transgenic rabbis with lymphocytic leukemia induced by the c-myc oncogene fused with the immunoglobulin heavy chain enhancer," PNAS USA, 85:3130-3134 (1988);

3. Vize et al., "Introduction of a porcine growth hormone fusion gene into transgenic pigs promotes growth," J. Cell. Sci., 90 (Pt 2):295-300 (1988); and

4. Mullins et al., "Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene," Nature, 344(6266):541-544 (1990).

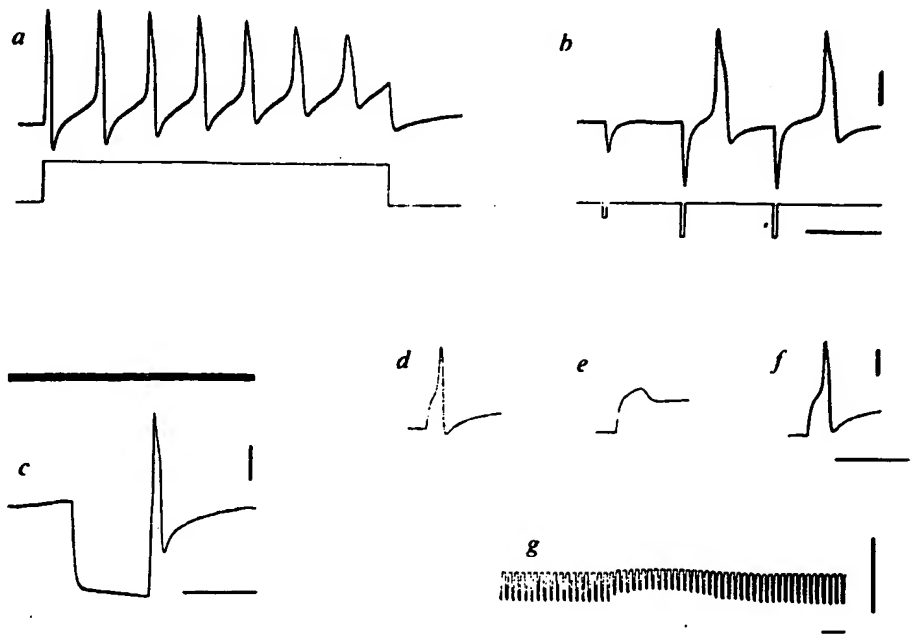

Fig. 2 Intracellular recordings from salivary gland cells of *H. ghilianii*. *a*, *b*, Action potentials, which may exceed 90 mV, are initiated by depolarizing current (*a*) and also following hyperpolarization by inward current (*b*; 100-ms pulses were applied). *c*, A hyperpolarizing current pulse in one salivary cell (lower trace) produces rebound excitation but there is no sign of electrical responses in an adjacent cell, recorded simultaneously at high gain on the upper trace. Experiments of this type indicate that the gland cells are not electrically coupled. *d*–*f*, Effect of 5 mM Co^{2+} added to the bathing solution. *d*, Control impulse in response to depolarizing current; *e*, 5 min after addition of $CoCl_2$ the action potential is abolished (a delayed rectification is apparent); *f*, recovery. This indicates that Ca^{2+} is the major current carrier for generation of action potentials. *g*, Brief application of 10^{-5} M serotonin produces a depolarization and increase in membrane conductance (indicated by reduction in amplitude of constant-current hyperpolarizing pulses). Voltage scales (vertical bars), 25 mV (1 mV in *c*, upper trace); time scales (horizontal bars), 2 s.

The natural stimulus for action potential generation, whether neural and/or hormonal, is unknown. Several putative neurotransmitters, however, (dopamine, serotonin and acetylcholine) were found to depolarize the gland cells, with an accompanying decrease in membrane resistance (Fig. 2*g*) and occasionally the production of no more than four impulses. Interestingly, in the presence of dopamine, applied depolarizing current was sometimes found to produce repetitive firing which was not simply a consequence of the depolarization produced by the drug. If the impulse provides a trigger for secretion, it seems unusual that the cells are normally so difficult to activate. Feeding, however, occurs very infrequently (every few months) and an action such as that of dopamine may mimic a natural process of bringing the gland into secreting condition.

In mammals, salivary and other exocrine gland cells are electrically inexcitable, producing graded potential changes (often hyperpolarizations) which may or may not be related to secretory function[10]. The *H. ghilianii* salivary cells are similar in their electrical excitability to mammalian endocrine cells such as those in the pancreas[12], adenohypophysis[13] and adrenal gland[14] (some molluscan exocrine glands produce action potentials[15]). This similarity extends to the anode-break excitation[8] shown by chromaffin[14] and anterior pituitary cells[13].

We have also found the *H. ghilianii* salivary gland to be suitable for molecular genetics because the cells have a very large ramifying nucleus that displays gene amplification of $\sim 10^6$ times; this should allow precise questions to be asked about the relationship between secretion and transcription/translation of identified genes. Thus, the *H. ghilianii* salivary gland, with its unusual combination of properties, represents a simple, accessible preparation with distinct experimental advantages for cellular studies of glandular secretion.

This specialized leech has been generally unavailable because in its natural habitat it is restricted to Amazonia. We have developed techniques for breeding this species and a facility has been set up by Biopharm to supply hementin to interested researchers.

Received 5 March; accepted 15 April 1985.

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Production of transgenic rabbits, sheep and pigs by microinjection

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Direct microinjection has been used to introduce foreign DNA into a number of terminally differentiated cell types as well as embryos of several species including sea urchin[1], *Candida elegans*[2], *Xenopus*[3], *Drosophila*[4,5] and mice[6–11]. Various genes have been successfully introduced into mice including constructs consisting of the mouse metallothionein-I (MT) promoter/regulator region fused to either the rat or human growth hormone (hGH) structural genes. Transgenic mice harbouring such genes commonly exhibit high, metal-inducible levels of the fusion messenger RNA in several organs, substantial quantities of the foreign growth hormone in serum and enhanced growth[12,13]. In addition, the gene is stably incorporated into the germ line, making the phenotype heritable. Because of the scientific importance and potential economic value of transgenic livestock containing foreign genes, we initiated studies on large animals by microinjecting the fusion gene, *MT–hGH*[13], into the pronuclei or nuclei of eggs from superovulated rabbits, sheep and pigs. We report here integration of the gene in all three species and expression of the gene in transgenic rabbits and pigs.

Studies with mouse ova indicated that integration of a gene into host chromosomes is much more efficient with nuclear than with cytoplasmic injection[14]. On this basis, we reasoned that nuclear injection would be an appropriate first approach with other species. The first problem encountered was visualization

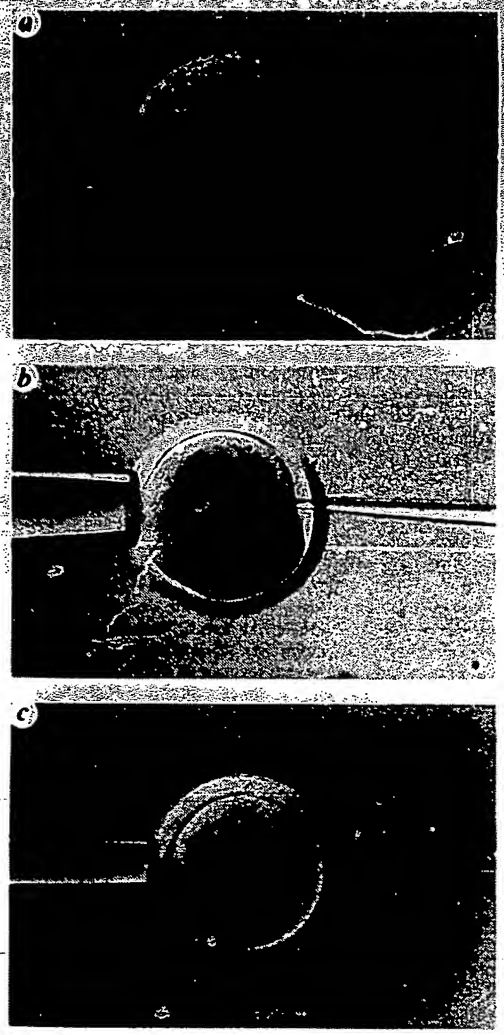

Fig. 1 Interference-contrast photomicrographs of one-cell fertilized ova from rabbit (*a*), sheep (*b*) and pig (*c*) following microinjection. Ova are held by a blunt holding pipette (diameter ~50 μm); an injecting pipette (diameter ~1.5 μm) has penetrated the zona pellucida, plasma membrane and pronuclear envelope. The tip is seen within the nucleoplasm immediately following injection of buffer containing DNA. The porcine ova (*c*) has been centrifuged at 15,000*g* for 3 min to reveal the normally obscure pronuclei[15]. Visualization of nuclear structures is aided by the use of interference-contrast optics, and microinjection is carried out under × 250 magnification using a Leitz microinjecting apparatus[10,14]. Injection was monitored by observing the diameter of the pronuclus or nucleus, which was expanded ~50%.

of the pronuclei or nuclei in the ova. Rabbit nuclear structures are readily seen (Fig. 1*a*). However, pronuclei and nuclei in sheep ova are difficult to locate and can only be seen by fluorescent microscopy using DNA specific fluorochromes (Hoechst, 33258) or by interference contrast (IC) microscopy (Fig. 1*b*). The combination of stain and ultraviolet light is damaging to the ovum (data not shown), so we used IC microscopy for microinjection. Fluorescent analysis indicated that IC microscopy is an effective method for pronuclear localization in approximately 80% of fertilized sheep eggs. Pig ova are opaque and no nuclear structures can be seen even with IC microscopy, but we found that centrifugation of pig ova at 15,000*g* for 3 min stratifies the cytoplasm (Fig. 1*c*), leaving the pronuclei or nuclei visible[15].

Once the nuclei could be visualized, microinjection was performed as described previously[10,14]. A few hundred copies of a 2.6 kilobase (kb) linear fragment containing the *MT-hGH* gene (see Fig. 2) were injected. Approximately 5,000 ova were injected and subsequently transferred to foster animals; about 500 of these resulted in fetuses or neonates (Table 1). The frequency of *MT-GH* integration was similar in the rabbit (12.8%) and the pig (11.0%) and low in sheep (1.3%). These integration efficiencies are probably accurate for the techniques being used because they are based on a large number of animals. The reasons for the lower integration frequency in these species compared to the mouse where it is ~27% are unknown but could be related to factors such as the concentration of DNA, buffer composition, age of the ovum and the structure of the chromosomes[14].

The number of copies of the *MT-hGH* gene that integrated was estimated by quantitative dot hybridization. Figure 2*d* shows the quantitation method as applied to transgenic pigs. Gene copy numbers ranged from 1 to 490 copies per cell (Table 2). The DNA from some of the transgenic animals was also analysed by restriction enzyme digestion of the chromosomal DNA followed by agarose gel electrophoresis and Southern blotting. Figure 2*a* shows the results obtained when the DNA was restricted with *Eco*RI, an enzyme that cuts once within the injected DNA. The probe detects two prominent bands in several rabbits and pigs. One band is close to the length of the injected DNA fragment (2.6 kb) and probably represents a tandem, head-to-tail array of the *MT-hGH* genes as is typically observed in transgenic mice[10,12]. The other band is approximately twice that length and might represent a head-to-head dimer, but further analysis will be required to test that possibility. When the DNA was restricted with *Sst*I (Fig. 2*b*), an enzyme that cuts twice within the injected DNA, two bands of the expected size were observed in all of the pigs and rabbits. Analysis of the sheep sample with *Eco*RI (not shown) and *Sst*I (Fig. 2*c*) revealed bands that were inconsistent with an intact *MT-hGH* gene, suggesting that the DNA had been trimmed or rearranged prior to integration.

Expression of the integrated genes was examined by quantitating *MT-hGH* mRNA by solution hybridization (Table 2). Only 4 of 16 rabbits analysed had any detectable *MT-hGH* mRNA in the liver, but the level was substantial in one of these. In mice, the frequency of expression of this gene is close to 70% (ref. 13). In pigs, mRNA levels were measured only in tail or ear samples because we did not want to risk adverse consequences of liver biopsy. Although tail and ear tissues are not primary sites of *MT* gene expression, we detected low levels of *MT-hGH* mRNA in several of the transgenic pigs (Table 2).

Plasma samples taken from pigs at birth and ~1 month later were analysed for hGH by radioimmunoassay. At birth, 11 of 18 pigs had detectable levels of hGH, ranging between 2 and 730 ng ml^{-1} (Table 2). One month later, hGH exceeded 300 ng ml^{-1} in three pigs. One rabbit also had a high level of hGH. Serum hGH as high as 64,000 ng ml^{-1} has been detected in transgenic mice, but accelerated growth rate was observed at levels of 20 to 80 ng ml^{-1} (ref. 13). None of these animals were exposed to high levels of zinc, a treatment that has been shown to activate *MT-hGH* gene expression ~10-fold in mice[13].

The effects of hGH on the growth of rabbits cannot be evaluated at present because only one live rabbit had detectable serum hGH and unfortunately it had malocclusion that impaired normal food consumption. Early indications are that the levels of hGH found in these transgenic pigs do not increase body weight dramatically. This may not be surprising considering that daily injections of bacterially synthesized hGH had no effect[16], and exogenous, highly purified porcine GH only stimulated growth by 10% when delivered during the major growth phase of the pig[17]. Transgenic offspring and littermate controls will need to be raised on normal and zinc-supplemented diets to determine precisely the effects of hGH on growth rate and other nutritional as well as endocrine parameters.

These experiments demonstrate that foreign genes can be introduced into several large animal species by microinjection of ova. Furthermore, expression of MT-hGH was obtained in rabbits and pigs. We used a fusion gene that has worked well in mice to demonstrate the feasibility of such techniques, and we are now trying several modifications in an effort to improve the level of expression and physiological response.

Table 1 Efficiency of producing *MT-hGH* transgenic rabbits, sheep and pigs by microinjection

Species	Transferred injected ova	Recipients	Integration frequency (%)	Expression frequency *MT-hGH* mRNA	Expression frequency Serum or plasma hGH
Rabbit*	1,907	73	28/218 (12.8)	4/16	1/1
Sheep†	1,032	192	1/73 (1.3)	ND	ND
Pig‡	2,035	64	20/192 (10.4)	11/20	11/18

Integration frequency is the number of animals (fetuses, stillborns and neonates) that retained the injected DNA/total number of animals resulting from injected ova. Six gilts bearing only injected eggs farrowed, producing 52 neonates, 5 of which retained DNA. In 31 gilts that farrowed, 204 fertilized control ova were transferred along with 859 injected ova to ensure sufficient embryos at implantation to maintain pregnancy. If survival of injected eggs to fetuses (16.4%) was similar for both groups, then injected eggs resulted in 140 of 252 fetuses and piglets produced, 15 of which retained injected DNA. We combined the data from the two groups to estimate integration efficiency. Expression frequency is the number of fetuses or neonates containing *MT-hGH* mRNA or plasma hGH per total number of animals examined. ND, not determined.

* Fertilized one-cell rabbit ova were flushed from the oviducts of superovulated New Zealand White (NZW) females 19 h after mating[20]. For microinjection the ova were placed in the well of a depression slide containing ~100 μl modified BMOC culture medium[21] with the $NaHCO_3$ replaced by 25 mM HEPES[22] and covered by silicone oil. Microinjection of embryos (1,857 one-cell and 50 two-cell) was performed as described for mouse ova[10,14]. Following injection, the ova were washed in fresh modified BMOC and surgically transferred to the oviducts of synchronized pseudopregnant rabbits[23].

† Rambouillet ewes were superovulated after exhibiting at least one prior oestrus period. On about day 10 of the oestrous cycle, progestagen-impregnated vaginal sponges (6α-methyl-17α-acetoxy progesterone, 60 mg; from Dr J. Lauderdale, Upjohn) were inserted and left for 12 days. Gonadotropin treatment (porcine follicle stimulating hormone, Burns) began three days before sponge removal and was continued twice daily (2.5 mg per injection, intramuscular) until the day following sponge removal[24]. At the onset of oestrus, ewes were either hand mated to fertile rams or inseminated *in utero* with 0.2 ml per horn of washed ram semen; 72 h after sponge removal, one-cell fertilized ova and cleaved ova were surgically collected from the reproductive tracts of anaesthetized ewes by flushing 6 ml Ham's F-10 medium containing 10% heat-inactivated fetal calf serum (FCS) from the utero-tubal junction through the cannulated infundibular end of each oviduct. The flushings were collected in sterile Petri dishes, and ova were removed under a dissecting scope. Ova were transferred to fresh Ham's F-10 containing 10% FCS and transported (~2.5 h) to Philadelphia in temperature-controlled containers. Microinjection of embryos (641 one-cell, 375 two-cell and 16 four-cell) was performed as previously described[10,14]. After embryos were injected, they were washed and transported to Beltsville. Embryos were aspirated into a glass micropipet tip with 10 μl Ham's F-10 and expelled 1-3 cm into the fimbriated end of the oviduct in synchronized recipient ewes. To assess the effects of transport and microinjection of DNA on egg development, a number of recipients bearing control and injected eggs were flushed 8 days following transfer. In recipients in which eggs were recovered, 26% of transported, uninjected and 10% of injected sheep eggs developed to blastocysts. Because of the high mortality of transported, injected eggs and the concern of multiple births, 5 or 6 embryos were transferred per recipient.

‡ Mature gilts were superovulated and bred as previously described[15]. At 18 to 27 h after the expected time of ovulation, gilts were anaesthetized and one cell fertilized the oviduct with 20 ml modified BMOC[21]. Ova were transferred to fresh BMOC and transported to Philadelphia. Microinjection of embryos (316 one-cell, and 1,719 two-cell) was performed as previously described[10,14]. The obscured pronuclei or nuclei or one- and two-cell pig ova were visible after centrifugation for 3 min at 15,000 *g*. Centrifugation of pig ova at this force and length of time has no detectable effect on development[15]. After embryos were injected, they were transported to Beltsville and transferred to the oviducts of recipient gilts as previously described[15]. To assess the effects of transport and microinjection of DNA on egg development, recipients bearing control and injected eggs were flushed 5 days following transfer. Approximately 52% of transported, uninjected and 23% of injected pig eggs developed to blastocysts. The pregnancy rate in recipients bearing only injected eggs was 50% while in recipients bearing both injected and control eggs 58% farrowed.

Table 2 Characteristics of transgenic rabbits, sheep and pigs

Species	Animal and sex	Gene copy (no. per cell)	*hGH* mRNA (molecules $cell^{-1}$)	Immunoassayable hGH ($ng\ ml^{-1}$)
Rabbit	59-3*	20	0	
	64-2♀	18	0	
	68-3†	28	39	ND
	68-4♀	24	0	
	122-9♀	11	0	
	131-8†	88	0	
	157-1♀	3	0	
	163-3♂	3	15	250
	167-5♀	10	0	
	179-1♀	16	0	
	179-2♂	36	0	
	179-5♀	6	0	
	200-3*	8	920	ND
	221-1*	40	140	ND
	223-4*	5	0	
	223-5*	40	0	
Sheep	693-1♀	1	ND	ND
Pig	100-3*	4	0	ND
	163-4*	140	0	ND
	3-2♂	330	26	Neg.
	3-6♂	490	53	17
	7-3♀	90	12	80
	10-4♀	23	0	Neg.
	11-2♂	1	0	40
	16-3♀	3	0	Neg.
	16-8♀	10	18	53
	16-9♂	1	5	65
	17-4♀	3	0	Neg.
	18-3♀	3	6	60
	20-2♂	2	4	40
	20-8♂	110	1	2
	21-4♂	1	2	Neg.
	21-5♀	1	0	Neg.
	22-1♀	50	24	56
	23-8♀	7	41	730
	25-2♀	17	0	108
	25-4♀	2	0	Neg

A 2.6-kb linear fragment of the fusion gene *MT-hGH*[13] containing the mouse MT-I regulator/promoter fused to hGH[19] was injected into fertilized one-cell and two-cell rabbit, sheep and pig eggs as described for mouse ova[10,14]. The male or female pronuclei of one-cell ova and both nuclei of two-cell ova were microinjected with a 3 ng μl^{-1} solution of DNA in Tris-EDTA buffer[14]. The ova were transferred into the oviducts of recipients at the same stage post oestrus as the donors (see Table 1). Animals without identified gender were either killed as fetuses(*) or were stillborn(†). The number of foreign fusion genes per cell was estimated by extracting total nucleic acids from a piece of fetal liver, neonatal ear or tail samples and performing quantitative dot hybridization with a 1.0-kb *Pvu*II probe spanning most of the *hGH* structural gene[19] (see Fig. 2). *MT-hGH* mRNA was measured by solution hybridization with a ^{32}P-labelled oligonucleotide (21-mer)[25]. For rabbits, either a partial hepatectomy was performed or fetal liver was used. For pigs, the *MT-hGH* mRNA content of ear or tail samples was quantitated. The concentration of *hGH* was measured in pig plasma obtained shortly after birth and serum from a 9-month-old rabbit. Samples were assayed in duplicate at 2.5 and 10 μl by radioimmunoassay using a hGH kit provided by Dr Raiti (National Hormone and Pituitary Program). The assay did not cross-react with porcine GH but required extra normal rabbit serum and anti-rabbit gammaglobulin to quantify hGH in rabbit samples. Pigs with hGH values less than 2 $ng\ ml^{-1}$ at birth were designated negative for hGH. At about one month of age, these pigs were also negative for hGH. ND, not determined; Neg, negative for hGH.

The key element in our success was the ability to visualize pronuclei and nuclei. Microinjection of the *MT-hGH* gene into the cytoplasm of 485 pig ova failed to produce DNA integration in 42 fetuses. Separate techniques for sheep and pigs were necessary because the opacity of the eggs differed. Although both contain dense cytoplasm, centrifugation did not help visualize pronuclei of sheep eggs and IC microscopy did not allow nuclear localization in pig ova. Preliminary work indicates

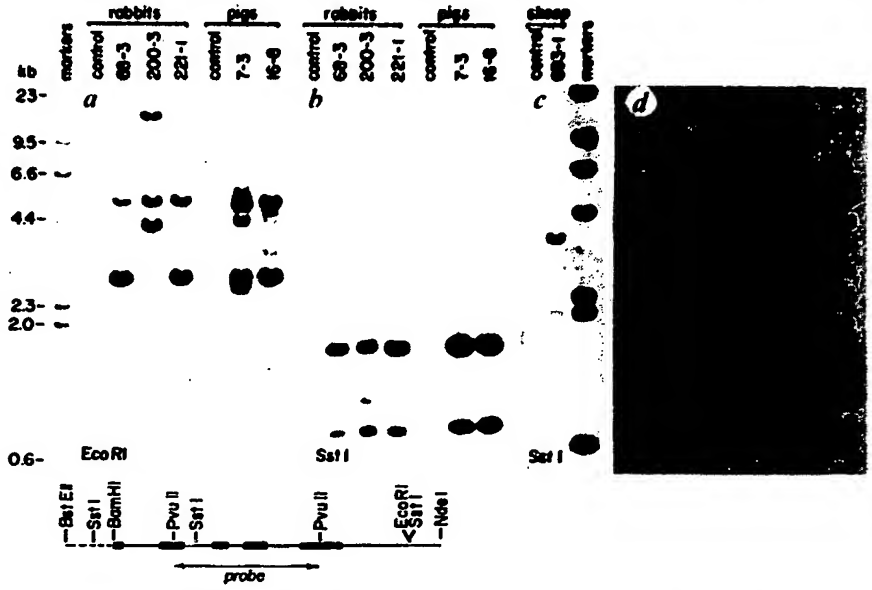

Fig. 2 Analysis of *MT-hGH* DNA introduced into rabbits, pigs and sheep. The diagram at the bottom shows the 2.6-kb *Bst*EII/*Eco*RI DNA fragment isolated from *MT-hGH* gene plasmid 111 that was microinjected[13]; the mouse MT-I promoter region is dashed, the *hGH* gene is solid, with the exons indicated as boxes, and the residual pBR322 sequences are dotted. The internal *Pvu*II fragment was isolated, nick-translated and used as a probe for quantitation of genes and Southern blots. Panels *a*, *b* and *c*: DNA (5 μg for controls and transgenic animals 200-3, 16-8 and 693-1; 1 μg mixed with 4 μg of control DNA for 68-3, 221-1 and 7-3) were digested with the indicated restriction enzymes (10 units, 6 h), electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with the nick-translated probe, washed and autoradiographed as described previously[18]. *a* and *b*, Exposure was 4.5 h; *c*, 24 h. For quantitation of gene copy number, 5 μg of DNA was spotted in duplicate onto nitrocellulose along with standards of 0, 0.5, 1, 2 and 5 μg of human DNA mixed with control DNA to make a total of 5 μg. *d* Shows the visualization *MT-hGH* gene copy number in transgenic pigs. After exposure, the spots were cut out and the radioactivity determined in a scintillation counter. Gene copy numbers were calculated from the standards assuming that the genome size of pigs and humans are comparable and that diploid human cells contain 10 genes homologous to the *Pvu*II fragment used as probe[19]. The results are shown in Table 2.

that these two techniques can be used for ova of other species; for example, IC microscopy allowed visualization of pronuclei in goat ova (unpublished observations) and centrifugation allowed localization of pronuclei in cow ova[15]. Although improvements in integration efficiency should be possible, the techniques have immediate application for both scientific and practical purposes.

We thank Mary Chandlee, Dennis McDuffie, Anne Powell, Leah Schulman, Myrna Trumbauer and Mary Yagle for technical assistance and Kenneth Bender, Paul Graninger, James Piatt, David Sherman and Stephanie Mengel for animal care. This work was supported in part by grants from the USDA (Section 1433 formula funds) and NIH (HD-19018) to R.L.B. and NIH (HD-09172) to R.D.P. R.E.H. was supported by an NIH training grant (HD-07155) and K.M.E. by an NIH postdoctoral fellowship (HD-06210).

Received 8 February; accepted 19 April 1985.

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Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells

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Haemophilia B, or Christmas disease, is an inherited X-chromosome-linked bleeding disorder caused by a defect in clotting factor IX and occurs in about 1 in 30,000 males in the United Kingdom[1]. Injection of factor IX concentrate obtained from blood donors allows most patients to be successfully managed. However, because of impurities in the factor IX concentrate presently in use, this treatment involves some risk of infection by blood-borne viruses such as non-A, non-B hepatitis and the virus causing acquired immune deficiency syndrome (AIDS)[2]. Because of the recent concern about the increasing incidence of AIDS amongst haemophiliacs, a factor IX preparation derived from a source other than blood is desirable. Here, we report that after introduction of human factor IX DNA clones[3] into a rat hepatoma cell line using recombinant DNA methods, we were able to isolate small amounts of biologically active human factor IX.

Factor IX is a plasma glycoprotein which has an essential role in the middle phase of the intrinsic clotting pathway[4] where, in an activated form, IXa, it interacts with factor VIII, phospholipid and calcium ions to form a complex that converts factor X to Xa. Factor IX is synthesized in liver hepatocytes where it undergoes three distinct types of post-translational modification before secretion into the bloodstream as a 415-amino-acid-long, highly modified protein. These modifications are the vitamin K-dependent γ-carboxylation of 12 glutamic acid residues[5], the addition of several carbohydrate residues[6] and the β-hydroxylation of a single aspartic acid residue[7]. The first two modifications are known to be required for activity of factor IX[5,6]. Because of the complex and specialized nature of these modifications, it seemed probable that the expression of active factor IX, derived from factor IX DNA clones, would be most likely to succeed in a hepatic cell or a transformed cell line derived from a hepatocyte. None of the standard mammalian hepatoma cell

Introduction of a porcine growth hormone fusion gene into transgenic pigs promotes growth

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Summary

Six transgenic pigs have been produced by microinjecting a human metallothionein promoter/porcine growth hormone gene construct into the pronuclei of fertilized eggs which were transferred to synchronized recipient sows. The resulting transgenic animals contained between 0·5 and 15 copies of the gene construct per cell, and at least one of the animals expressed the introduced gene and grew at an increased rate compared to both transgenic and non-transgenic littermates. Some of the transgenic animals that did not appear to grow at increased rates were found to contain rearranged gene sequences. Two of the transgenic pigs have been shown to pass on the introduced genes to their offspring.

Key words: transgenic pigs, gene rearrangement, growth hormone, growth improvement.

Introduction

Transgenic mice containing integrated copies of a wide range of different genes have been produced (Palmiter & Brinster, 1986). These animals, and their transgenic offspring, usually express the introduced transgenes in a manner appropriate to the promoter/enhancer utilized in the introduced gene. When the expressed transgene encodes a growth promoting hormone, such as growth hormone or growth hormone releasing factor, transgenic mice can grow up to twice normal size (Palmiter *et al.* 1982*a*,*b*, 1983; Hammer *et al.* 1985*a*). The application of this approach to improving the growth of livestock has been hindered by technical difficulties encountered in producing transgenic farm animals, and techniques that enable the generation of transgenic sheep and pigs have only recently become available (Hammer *et al.* 1985*b*). The introduction of a fusion gene containing the human growth hormone gene linked to a murine metallothionein promoter into transgenic rabbits, sheep and pigs has previously been demonstrated (Hammer *et al.* 1985*b*). Although some of these animals were shown to express the introduced gene by both RNA and protein analysis, none of them grew at enhanced rates. In this report we describe the production of transgenic pigs using a gene construct, which contains a porcine growth hormone (pGH) fusion gene under the transcriptional control of the human metallothionein-IIA (hMT-IIA) promoter. This construct has previously been shown to markedly enhance growth rates when introduced into the germ line of transgenic mice (unpublished results).

Materials and methods

DNA manipulations

All DNA manipulations involved well-established techniques (Maniatis *et al.* 1982). Plasmid pHMPG.4 was constructed by inserting a *Hin*dIII–*Eco*RI fragment containing hMT-IIA promoter sequences from −765 to +60 (Karin & Richards, 1982) into pUC19. This plasmid was then digested with *Eco*RI and ligated to the 800-bp *Eco*RI insert of the full-length pGH cDNA clone pPG.3 (Vize & Wells, 1987*a*). Of the resulting plasmids, one which contained the *Eco*RI fragment in the correct orientation was selected. This clone was then digested with *Sma*I, and ligated to the Klenow-treated 1-kb *Sma*I–*Bam*HI fragment from cosmid cPGH.1, which contains the 3′ end of the pGH gene (Vize & Wells,

Journal of Cell Science 90, 295–300 (1988)

1987*b*). The resulting clones were screened by restriction mapping and a clone that contained the fragment inserted in the desired orientation identified and named pHMPG.4 (HM, human metallothionein; PG, porcine growth hormone).

The DNA fragment used for microinjection was a 2·7-kb *Hin*dIII–*Pvu*I fragment isolated from pHMPG.4. Following digestion with these two restriction enzymes the desired fragment was separated from other reaction products by electrophoresis through a low melting temperature agarose gel, and purified by one phenol followed by one phenol: chloroform (1:1) extraction and ethanol precipitation. The purified fragment was resuspended in 10 mM-Tris·HCl (pH 7·5), 1 mM-EDTA and diluted to a concentration of 2 ng μl^{-1} with phosphate-buffered saline for microinjection.

Southern blots (Southern, 1975) were performed using Zeta-probe membranes and the alkaline transfer procedure of Chomczynski & Qasba (1984) as modified by Reed & Mann (1985). Dot and slot-blots were performed using GeneScreen membranes under the conditions described by the manufacturer. In all cases the hybridization probe was a nick-translated (Maniatis *et al.* 1982) *Hin*dIII–*Ava*I fragment from the 5′ end of the human metallothionein-IIA promoter (Karin & Richards, 1982).

Generation of transgenic animals

Multiparous Large White sows were stimulated with 750 IU PMSG (Folligen Intervet) followed by 500 IU hCG 44 h later. Sows in oestrus were hand mated to a fertile boar and slaughtered for egg collection 10 h after the expected time of ovulation. The fertilized pig eggs were then recovered from the reproductive tracts of the slaughtered animals. The cytoplasm of eggs was stratified by centrifugation (7000 *g* for 3 min) to allow the visualization of pronuclei (Hammer *et al.* 1985*b*), and one of the pronuclei injected with 2 pl of the pHMPG.4 insert (containing approximately 600 copies of the 2·7-kb linear DNA fragment). Following overnight culture (Stone *et al.* 1984) the surviving eggs were surgically transferred to the oviducts of synchronized recipient sows.

Results

The organization of the gene construct used in these experiments, pHMPG.4, is illustrated in Fig. 1. This plasmid contains the hMT-IIA promoter (Karin & Richards, 1982) fused to a hybrid pGH gene consisting of pGH cDNA sequences (Vize & Wells, 1987*a*) encoding amino acid residues 1–158 of pre-pGH, and pGH genomic sequences (Vize & Wells, 1987*b*) encoding pre-pGH residues 158–216. The genomic sequence also contains approximately 700 bases of pGH gene 3′ non-coding sequences. The 3′ non-coding sequences were included to ensure the efficient processing and polyadenylation of the hybrid messenger RNA. The 2·7-kb *Hin*dIII–*Pvu*I insert of pHMPG.4 was purified and used for microinjection into fertilized pig eggs.

In a preliminary series of experiments, 189 single-cell embryos, which had each been injected with approximately 600 copies of the plasmid insert, were transferred to 13 recipient sows, at an average of 13 embryos per sow. Four of the surrogates became pregnant as assessed by estrone sulphate determination (Stone *et al.* 1986) but none farrowed. In a second series of transfers, a total of 423 microinjected embryos were transferred to 14 synchronized recipient sows, this time at an average of 30 per sow. Six of the recipients returned to oestrus during the fourth week following the transfer, and four had to be culled because of vaginal bacterial infection (not connected with the embryo transfer procedure). The remaining four sows completed the pregnancy and produced a total of 17 piglets. Thus, the frequency of producing piglets from injected embryos was one live birth per 16 embryos excluding the embryos from infected sows. This is similar to the efficiency obtained with mouse

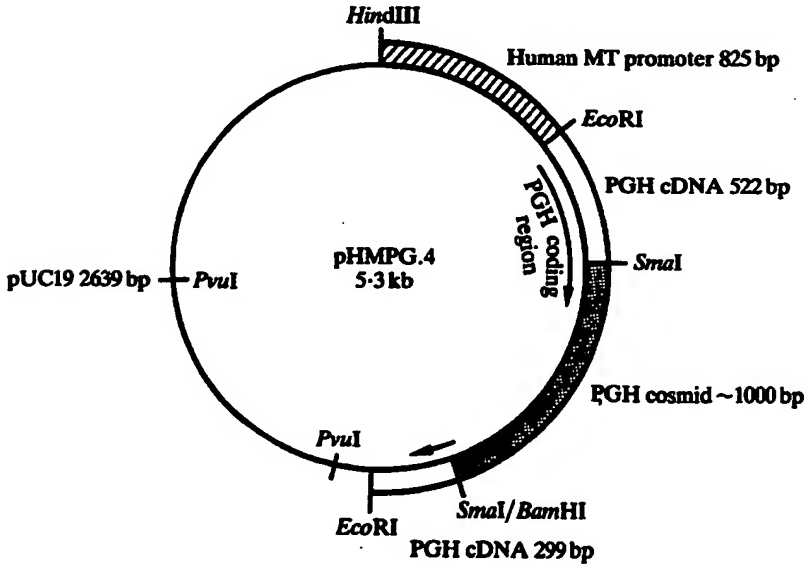

Fig. 1. Structure of plasmid pHMPG.4. The construction of this plasmid is described in detail in Materials and methods. The plasmid contains 765 bp of promoter and 60 bp of 5′ untranslated sequence from the human metallothionein-IIA promoter (Karin & Richards, 1982) linked to a hybrid pGH gene constructed by fusing pGH cDNA (Vize & Wells, 1987*a*) and pGH cosmid (Vize & Wells, 1987*b*) sequences. The *Hin*dIII and *Pvu*I restriction sites used to excise the hMT-IIA/pGH fusion gene are indicated.

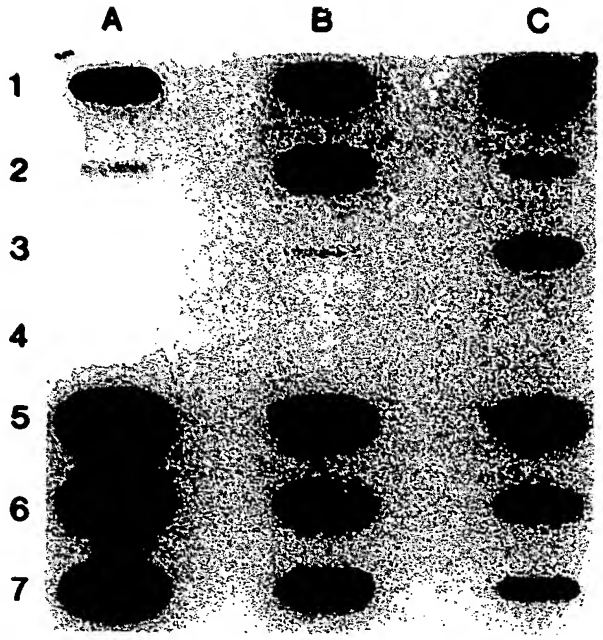

Fig. 2. Gene copy number in transgenic pigs. DNA samples from transgenic pigs were examined by slot-blot analysis. Samples were filtered onto a membrane using a slot-blot apparatus, which also contained pig negative and human positive controls, and plasmid DNA samples corresponding to known gene copy numbers. The samples (5 μg) are: row 1, transgenic pigs 177, 180 and 295; row 2, transgenic pigs 375, 736 and 739; row 3 (no sample in first column) pig negative control and human positive control; row 5, plasmid standards corresponding to 40, 10 and 4 gene copies per cell; row 6, as for row 5 except corresponding to 30, 8 and 2 gene copies per cell; row 7, as per row 6 except amounts correspond to 20, 6 and 1 gene copies per cell.

one-cell embryos injected with the same plasmid construct, where we achieved one mouse pup from an average of 11 injected embryos.

Dot-blot analysis was performed on DNA isolated from tail tissue (Palmiter *et al.* 1982*a*) from each of the 17 piglets produced, and revealed that six of the animals that developed from injected eggs contained sequences homologous to the hMT-IIA promoter hybridization probe, with copy numbers (determined by slot-blot analysis) ranging from 0·5 to 15 copies of the pHMPG.4 insert per cell (Fig. 2). The two animals with less than one copy per cell (see Table 1) will not be discussed further.

Southern analysis was performed on the DNA of each transgenic pig to determine the chromosomal organization of the introduced sequences. Plasmid pHMPG.4 does not contain *Bam*HI restriction sites, so the digestion of transgenic animal DNA with this enzyme will produce one band per integration site on Southern analysis. The data show that each of the transgenic pigs contains a single band, which hybridizes to the hMT-IIA promoter probe (Fig. 3A). In three cases, (pigs 180, 295 and 736) the detected bands are of greater than 25 kb in length, and so appear to be of identical size. Longer gels using lower percentages of agarose also produce a similar result to that seen in Fig. 3A, and we therefore conclude that each of these four animals probably contains the transgenes integrated into a single chromosomal site in each case.

*Eco*RI cuts twice within the pHMPG.4 insert, so digestion with this enzyme followed by hybridization to hMT-IIA sequences can identify the presence of head-to-head multimers, head-to-tail multimers, and flanking *Eco*RI restriction sites (Fig. 1). Digestion of DNA from each of the transgenic pigs with *Eco*RI followed by Southern analysis (Fig. 3B) revealed that only one animal, pig number 295, produced a pattern of bands that indicated that all integrated copies of the pHMPG.4 insert were in a tandem head-to-tail array. Of the remaining three pigs, one, number 177, showed a random arrangement (or possibly tail-to-tail), while the remaining two animals produced multiple bands indicative of rearranged sequences.

Serum pGH concentrations (Table 1) were determined for each of the transgenic and control pigs using an ELISA assay system (Signorella & Hymen, 1984) to determine if the introduced genes had produced any effect on serum growth hormone levels. One of the transgenic pigs, number 295, contained an elevated level of serum pGH, which was over twice that of non-transgenic and transgenic littermates both at birth and at 50 days post-partum (Table 1).

All piglets were monitored under conventional commercial rearing conditions with *ad libitum* feeding. Growth was monitored by weighing at weekly intervals. No differences were evident in the growth rate of piglets up to 20 kg live weight. Subsequently, one of the two transgenic sows, number 177, continued to grow at a similar rate to non-transgenic control littermates, while the other, number 295, began to grow substantially faster (Table 1), and had achieved the target market weight of 90 kg at 17 weeks of age as opposed to 22–25 weeks required by her littermates. It is difficult to judge if either of the two transgenic males possess enhanced growth rates as only two non-transgenic male controls were produced in the four litters. Neither of the transgenic males showed evidence of markedly increased serum pGH levels (Table 1). Following breeding (see below) the largest of the transgenic boars, number 180, was sacrificed at 35 weeks of age, and a number of tissues examined for the presence of pGH mRNA by both Northern and nuclease protection analysis. No expression of the introduced gene was detected in liver, kidney, spleen, brain, testis or pituitary (data not shown).

Table 1. *Weight gain and serum pGH levels in transgenic pigs*

Animal	Sex	No. of transgenes per cell	Plasma pGH (mIU ml^{-1})		Weight gain (g day^{-1})
			At birth	At 50 days	
177	F	3	2·5	10·4	758
180	M	6	4·2	15·3	845
295	F	15	27·8	27·8	1273
375	F	0·5	6·3	ND	680
736	M	6	1·1	11·1	646
739	F	0·5	0·5	6·9	700
Non-transgenic littermates	F + M	–	6·4 ± 5·2	11·3 ± 2·7	781 ± 44

The mean daily weight gain was determined between 20 and 90 kg live weight. F, female; M, male; ND, not determined.

Considering the high incidence of gene rearrangement in the founder transgenic pigs, it was important to demonstrate that, once incorporated, the introduced genes could be stably transmitted to offspring. Two of the transgenic pigs, one with a random arrangement (female 177) and one with rearranged (male 180) sequences, were therefore mated with control animals, and the offspring examined for the presence of the foreign gene by dot-blot analysis. As Fig. 4 illustrates, both of the animals were able to pass on the transgene to a proportion of their offspring (pig 177, one out of five; pig 180, six out of eight), indicating that the generation of lines of transgenic pigs is feasible. Southern analysis of DNA from each of the transgenic offspring revealed that each of the pigs produced restriction enzyme patterns identical to those of their transgenic parent (data not shown).

Discussion

The finding that only one of the four transgenic pigs studied contained the introduced genes organized in a head-to-tail array is surprising, as all seven transgenic mice containing the same gene construct, which have been studied by Southern blotting, contain the transgenes in head-to-tail arrays (for example, see McInnes *et al.* 1987). Also, the results of a large number of other investigators indicate that nearly all transgenic mice contain integrated transgenes in this conformation (for reviews see Palmiter & Brinster, 1985, 1986; Gordon &

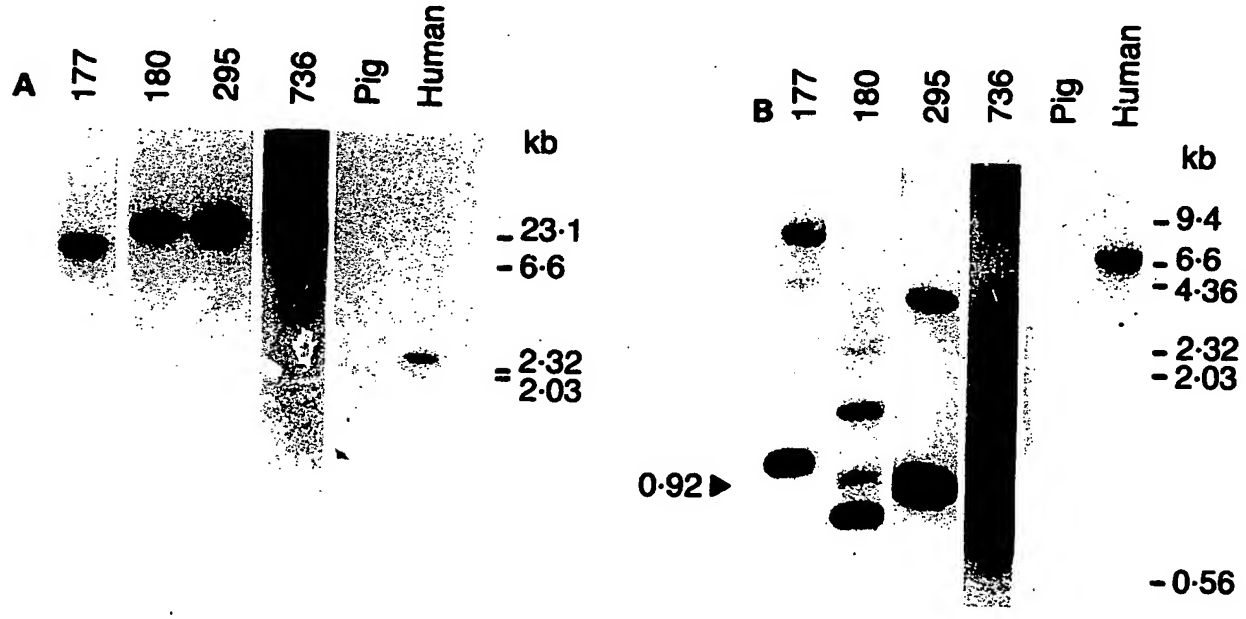

Fig. 3. Southern analysis of transgenic pigs. DNA samples (3 μg) from each of the transgenic pigs along with pig negative and human positive controls was digested with either *Bam*HI (A) or *Eco*RI (B), electrophoresed through an agarose gel followed by transfer to a membrane and hybridization to a nick-translated human metallothionein-IIA promoter probe. The 0·92-kb band (pig 295) expected from *Eco*RI digestion of a head-to-tail array is indicated in B. Molecular weight markers (in kb) are shown on the right of each gel.

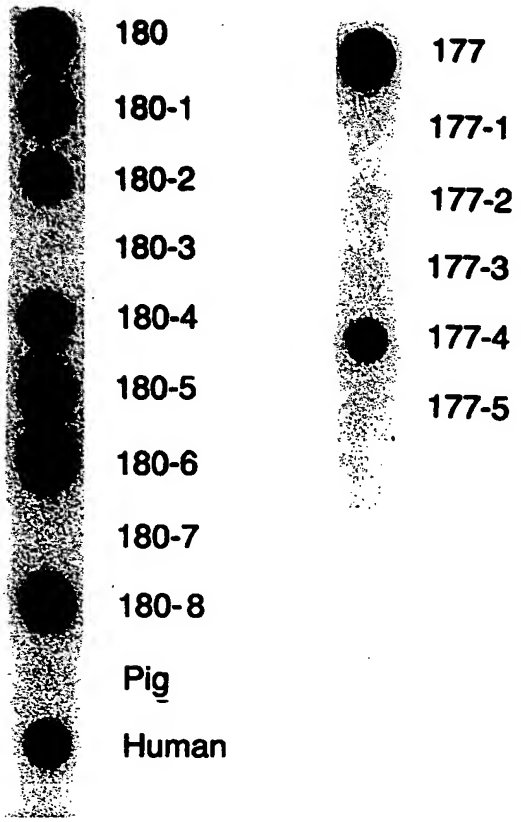

Fig. 4. Inheritance of the transgene. DNA samples (5 μg) from the two transgenic parents along with that of their offspring, plus appropriate negative (pig) and positive (human) controls, were applied to a membrane using a dot-blot apparatus and hybridized to a nick-translated human metallothionein-IIA promoter probe. Pig 180, and offspring 177-1, 177-2 and 180-1 to 180-3 were male, and pig 177 and offspring 177-3 to 177-5 and 180-4 to 180-8 female. Despite appearances here, Southern analysis of pigs 177 and 177-4 indicates that both of these animals contain an equal number of gene copies per cell (data not shown).

Ruddle, 1985). The only available data on integration patterns in transgenic pigs other than those presented here are those of Hammer *et al.* (1985*b*), who have shown that in the two transgenic pigs studied the DNA was integrated in an ordered fashion and inserts were found mostly in head-to-tail arrays. As the mouse data indicate that our construct is not inherently unstable, and the results of Hammer *et al.* (1985*b*) indicate that other constructs integrate in a similar fashion in the porcine and murine genomes, we concluded that the rearrangement of the pHMPG.4 sequences in transgenic pigs is probably a phenomenon specific for this construct in the porcine genome.

The data presented here demonstrate for the first time that the expression of a transgene in a transgenic farm animal can result in markedly improved daily weight gain and body weight. As transgenic pigs expressing high levels of human growth hormone do not appear to grow at increased rates (Hammer *et al.* 1985*b*), we propose that the enhanced growth of our transgenic female number 295 is due to the use of a gene construct that directs the expression of authentic porcine growth hormone, rather than a heterologous hormone. It is interesting to note that the only pig showing evidence of improved growth performance was also the only animal that contained the introduced gene sequences integrated in a random head-to-tail array. The increased serum pGH and improved growth performance of this animal did not result in any detrimental effects on health, and there were no indications of liver damage or arthritis as are observed after prolonged exposure to high levels of injected pGH (Machlin, 1972). This is probably due to this animal expressing growth hormone at less than toxic levels (Chung *et al.* 1985). Unfortunately, this animal contracted pneumonia following an uncommonly severe period of cold weather and was killed at 18 weeks of age, precluding studies on the fertility of this animal or its ability to transmit its transgenes to offspring. Such studies are of great importance, as transgenic female mice expressing high levels of human growth hormone are infertile (Palmiter *et al.* 1983). This may not be as severe a problem in pHMPG.4 transgenic pigs, as these animals will be expressing porcine growth hormone, not a heterologous growth hormone, and transgenic mice with elevated murine growth hormone levels do not display any indications of reduced fertility (Hammer *et al.* 1985*a*). However, the study of the effect of increased pGH levels in transgenic pigs will have to await the production of a large number of transgenic pigs containing functional transgenes.

Our results also demonstrate for the first time that transgenes integrated into the procine genome are stable in the germline, and can be passed onto a proportion of the offspring, indicating that the production of stable lines of transgenic farm animals with enhanced growth performances is feasible.

Our future research is aimed at the generation of a larger number of transgenic pigs using the pHMPG.4 construct and generating homozygous lines of transgenic pigs from these founder animals to determine the potential of this approach for improving pig production. Studies on the activity of the hMT-IIA promoter with transgenic animals fed on diets containing increased levels of heavy metals (such as zinc) are also planned as transcription rates from this promoter are enhanced by heavy metals (Karin *et al.* 1984). Experiments aimed at determining the region of the pHMPG.4 construct responsible for the observed instability in the porcine genome are also in progress.

This work was supported by grants from the Commonwealth Research Centre for Gene Technology and the

Australian Pig Industry Committee. P.D.V. was the recipient of a Commonwealth Postgraduate Research Award and an Australian Pig Industry Research Committee Special Research Fellowship, A.E.M. was the recipient of a University of Adelaide Research Award. We would like to thank Dr L. S. Coles for critical discussions on the manuscript.

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(Received 20 October 1987 – Accepted, in revised form, 4 January 1988)

families (solid line) and acute families (dotted line). The peak multipoint lod score for chronic SMA is 9.03, and the peak lod score for acute SMA is 2.02. Pairwise lod scores for chronic and acute SMA families versus four markers located in the middle of the linkage region are shown in Table 1. The maximum two-point lod score for chronic families is 8.43 at a recombination fraction of 2% with marker D5S6, and 1.71 for acute families at a recombination fraction of 2% with marker D5S78.

Application of the HOMOG program[13] to the multipoint lod scores of the families with chronic SMA gave no evidence for heterogeneity among these families. Although the power of homogeneity tests can be lower in recessive families than in larger families with dominant diseases, the absence of evidence for heterogeneity led us to adopt the most parsimonious solution of assuming homogeneity. The confidence interval for the location of the gene for chronic SMA is 11 centimorgans (cM) wide and spans a region 2 cM proximal of locus D5S6 to a point 4 cM proximal of locus D5S78 (note arrows in Fig. 1). For families with acute SMA, the maximum lod score of 2.02 indicates that a gene responsible for this disease maps to the same general area. The best estimate for the location of the acute SMA locus is 15 cM distal to the estimated position of the locus for chronic SMA.

Our data indicate that clinically heterogeneous forms of chronic childhood SMA (type II or intermediate form and type III or Kugelberg–Welander or mild form) map to a single locus on chromosome 5q. The chronic forms of childhood-onset SMA, therefore, are likely to occur as the result of allelic heterogeneity, similar to the case for Duchenne- and Becker-type dystrophies[15]. It is interesting that our data indicate that acute childhood SMA (type I or Werdnig-Hoffmann or infantile SMA or severe SMA) map to the same, or a closely linked, locus on 5q. Other informative acute families must be analysed to confirm the linkage of this form of SMA and to evaluate the associated map location relative to that of chronic SMA. Also, other chronic families must be analysed to further assess the possible occurrence of nonallelic heterogeneity. It will be interesting to determine whether adult-onset and dominantly inherited cases of SMA similarly map to chromosome 5q. The gene encoding hexosaminidase B maps between markers D5S39 and D5S78 (refs 16, 17). Deficiencies in both the α- and β-subunit of this enzyme have been associated with chronic cases of SMA[18,19]. We are investigating whether this gene is a candidate for an SMA mutation. □

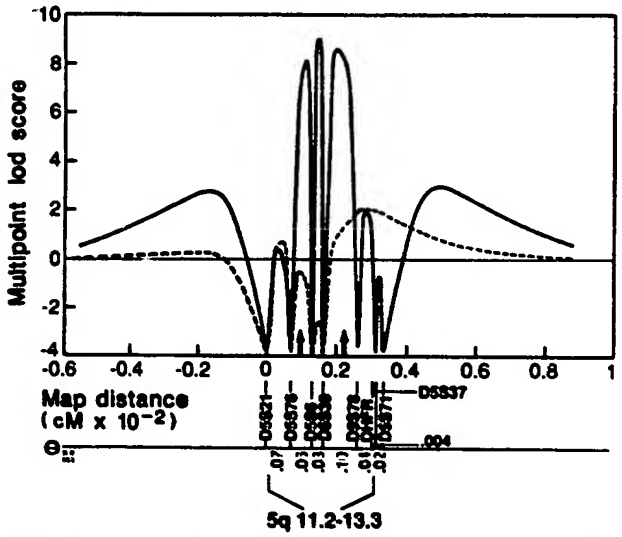

FIG. 1 Multipoint linkage analysis of the SMA disease locus with eight DNA markers spanning ~30 cM, including 5q11.2–5q13.3 (refs 16, 21). Analysis of seven chronic families (solid line) and six acute families (dotted line). Three chronic families each consists of four affected children and 8–12 unaffected sibs. Four chronic families each have three affected children and 0–4 unaffected sibs. The acute families, collected over a 3-year period, include one family with three affected children (trizygotic triplets), four families with two affected children, and one family with one affected and two unaffected sibs. Recombination fractions (θ_M) between DNA markers were calculated from published map distances[16]. Marker loci D5S6, D5S39, D5S78 and DHFR map to 5q11.2–13.3 (ref. 21). For the female-to-male distance ratio we used the published value of 1.6 as being appropriate for this area of the genome[22]. Multipoint lod scores were obtained by five-point analysis in all families, except one for which, for reasons of computational efficiency, three-point lod scores had to be calculated. The computer program used was LINKMAP of the LINKAGE package[20]. The confidence interval for chronic families (defined as points on the map with lod scores $\geq Z_{max}^{-1}$ where Z_{max} is the value of $Z(\theta)$ at the maximum likelihood estimate of θ) spans an 11-cM region marked by arrows at map positions 0.11 and 0.22.

Received 11 January; accepted 16 February 1990.

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ACKNOWLEDGEMENTS. We are indebted to the SMA families whose cooperation and support made this project possible, and also to the early contributions of Dr Michael Mandelstehn. Thanks to Barbara Byth, Dr Sally Candy, and Bartolome Jaume-Roig for technical assistance, Linda Sherry for family coordination, Dr Kamma Das for tissue culture, and Dr Lewis P. Rowland for helpful discussions. This work was supported by the Muscular Dystrophy Association of America, the Muscular Dystrophy Group of Great Britain and Northern Ireland, the MRC of Great Britain, and the W. M. Keck Foundation.

Fulminant hypertension in transgenic rats harbouring the mouse *Ren-2* gene

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PRIMARY hypertension is a polygenic condition in which blood pressure is enigmatically elevated; it remains a leading cause of cardiovascular disease and death due to cerebral haemorrhage, cardiac failure and kidney disease. The genes for several of the proteins involved in blood pressure homeostasis have been cloned and characterized[1–8], including those of the renin–angiotensin system, which plays a central part in blood pressure control[9–10]. Here we describe the introduction of the mouse *Ren-2* renin gene[3,11–13] into the genome of the rat and demonstrate that expression of this gene causes severe hypertension. These transgenic animals represent a model for hypertension in which the genetic basis for the disease is known. Further, as the transgenic animals do not overexpress active renin in the kidney and have low levels of active renin in their plasma, they also provide a new model for low-renin hypertension.

We chose the mouse *Ren-2* renin gene for introduction into the rat germline because it had already been characterized in transgenic mice and because we expected it to be highly

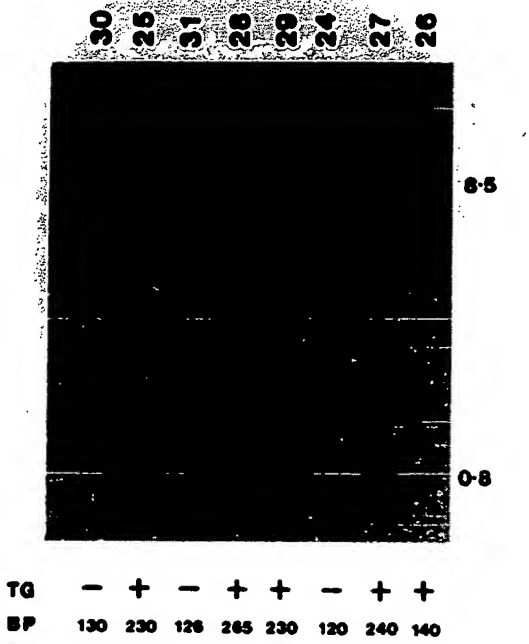

FIG. 1 Southern blot identifying animals carrying the DBA/2 *Ren-2* gene. The identification numbers of potential founder animals are shown above the corresponding lane and the positions of the *Ren-2*-specific 8.5-kb and 0.8-kb restriction fragments are indicated to the right. Transgenic (TG) positive and negative animals are indicated by symbols under the corresponding lane, together with the systolic blood pressure (BP, in mm Hg) of each animal at the age of 10 weeks.
METHODS. DNA preparation: DNA was prepared from tail biopsies and digested with *Pvu*II. After electrophoresis on a 0.8% agarose gel, samples were Southern-blotted and hybridized with a ^{32}P-labelled dCTP 300-bp probe derived from the renin complementary DNA clone pDD1D2[17] and labelled by random priming[18]. Preparation of transgenic animals: DNA was prepared for microinjection by digestion of the cosmid clone cosDBA-1 (ref. 17) with *Xho*I, and subsequent isolation of the 24-kb *Xho*I fragment containing the *Ren-2* gene on a 10–20% sucrose gradient in 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM sodium acetate. Fractions containing the required fragment were pooled and recovered by ethanol-precipitation before being centrifuged on a CsCl gradient[19]. DNA was diluted to a final concentration of 1 μg ml^{-1} in injection buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA), and stored in aliquots at −20 °C before use. Fertilized eggs were derived from a cross between Sprague-Dawley female and WKY male rats after superovulation of immature females (at 4 weeks old) according to the procedure of Armstrong *et al.*[20]. Eggs were cultured, microinjected, and re-implanted as described for the mouse[19]. Rats were all bred in our own facilities.

expressed in certain tissues[14]; also, injection of purified mouse submandibular gland (SMG) renin (encoded by *Ren-2*) into rats leads to a significant and sustained increase in blood pressure[15]. Fertilized rat eggs were microinjected with a linear DNA fragment containing the entire DBA/2J *Ren-2* gene, including 5.3 and 9.5 kilobases (kb) of 5′ and 3′ flanking sequence, respectively[14]. From 37 eggs implanted, there were eight progeny, of which five carried the transgene (Fig. 1). Four of the founders were bred successfully and three of them (TGRmRen2, numbers 25, 26 and 27) transmitted the transgene to their progeny. At ten weeks of age and before breeding, the blood pressure of the founder animals was measured. For four of the transgenic animals it was in the range 230–265 mm Hg, but was 120–130 mm Hg in the transgene-negative litter-mates (Fig. 1). Breeding of TGRmRen2 female 26, who was not hypertensive, revealed her to be mosaic for a transgene insertion site, the inheritance of which segregated with hypertension in the blood pressure range indicated (data not shown). The phenotype is therefore independent of the transgene insertion site and is not due to a fortuitous mutation associated with the integration event.

Analysis of the transgenic line established from TGRmRen2 male 27 revealed that, without exception, progeny inheriting the transgene also had the hypertensive phenotype. Both male and female animals of this line developed hypertension rapidly, beginning at four weeks of age and reaching a maximum by nine weeks (Fig. 2*a*). Pharmacological intervention to reduce blood pressure took the form of treating the animals with 10 mg kg^{-1} per day of the converting enzyme inhibitor, captopril; this inhibits the conversion of angiotensin I to angiotensin II. This low dose, given daily in the drinking water, was sufficient to reduce the blood pressure of the hypertensive transgenic rats reproducibly by 40–60 mm Hg (Fig. 2*b*), indicating that the hypertension is largely dependent on the conversion of angiotensin I to angiotensin II.

Northern blot analysis showed that the concentration of renin transcripts was high in the adrenal glands of the transgenic animals (Fig. 3*a*). In addition, renin transcripts were detectable in testis, coagulation gland, thymus and small intestine in transgene-positive animals, but not in control transgene-negative littermates (data not shown). These additional sites represent tissues in which renin is naturally expressed in the mouse. Renin messenger RNA was not observed in the SMG, a result that could reflect the absence of essential *trans*-acting factors in this tissue as the endogenous rat renin gene is not expressed in the SMG (ref. 16). An RNase protection assay using a *Ren-2*-specific probe confirmed that the renin transcripts in the adrenal gland were exclusively of *Ren-2* origin and that *Ren-2* transcripts were present in the kidneys of transgene-positive animals (Fig. 3*b*).

No evidence was found for altered plasma angiotensinogen levels, but plasma renin activity and angiotensin I were significantly lower in transgenic animals than in the controls (Fig. 4*b*–*e*). The amount of angiotensin II was also less than in the

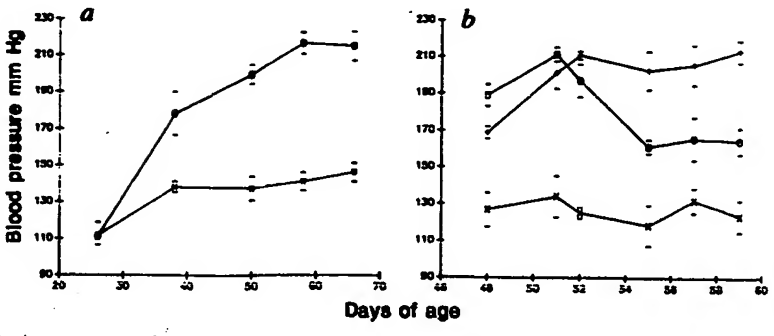

FIG. 2 *a*, Development of blood pressure with age. Each point represents the mean of 7 (transgenic, circles) or 5 (control, crosses) animals and standard errors are indicated above and below each data point. *b*, Effect of converting enzyme inhibitor (CEI) on blood pressure. Each point represents the mean of 3 animals and standard errors are indicated above and below each data point. +, TGRmRen2 L27 rats having no treatment; O, TGRmRen2 L27 rats receiving CEI; ×, control rats receiving CEI.
METHODS. Blood pressure was determined by tail plethysmography under light ether anaesthesia as described[21]. Animals under converting enzyme inhibitor treatment were given captopril (10 mg kg^{-1} per day) in their drinking water. Captopril treatment started at day 51.

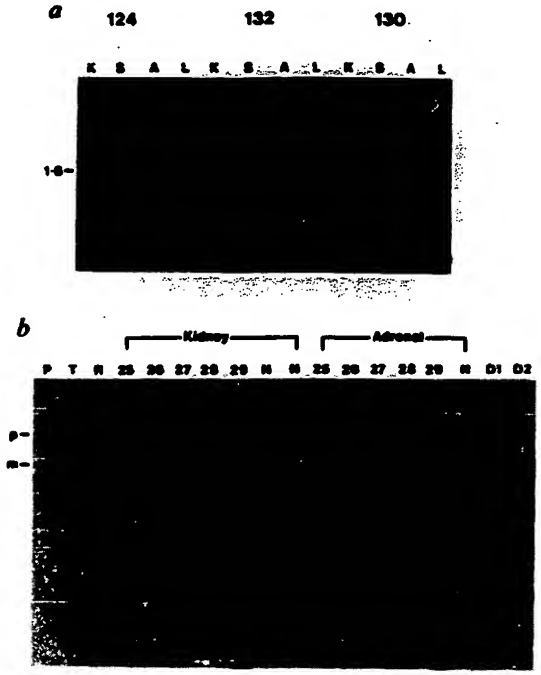

FIG. 3 Northern blot and RNase protection assay. a, Northern blot of RNA isolated from the kidney (K); SMG (S); adrenal gland (A); and liver (L) of transgene-positive (124 and 130) and transgene-negative (132) male rats. The size of the hybridizing RNA is indicated in kb. With the exception of the adrenal gland (5 μg), 40 μg total RNA was used for each sample. b, RNase protection of kidney and adrenal gland RNA from transgenic rats (numbers 25–29) and control littermates (R). The following controls are included: P, undigested probe; T, transfer RNA (9 μg); R, rat kidney RNA (20 μg); D1 and D2, mouse (DBA/2J) kidney RNA (20 μg and 40 μg, respectively). The positions of the undigested 244-nucleotide probe (p) and the 224-nucleotide mouse-specific protected fragment (m) are indicated.

METHODS. Preparation of RNA: Total RNA was isolated from mature rats as previously described[3] or by homogenization in guanidine isothiocyanate.[22] Northern blot analysis: Northern blots were prepared and hybridized as previously described[23] with a ^{32}P-labelled renin cDNA probe (pDD1D2) by random priming[18] and washed with 0.1 × SSC, 0.1% SDS at 65 °C. RNase protection assay: ^{32}P-labelled RNA transcripts were prepared by transcription of a 244-nucleotide antisense RNA from the plasmid pSLM (ref. 15) using SP6 RNA polymerase. This transcript comprised 224 nucleotides of *Ren-2* antisense RNA and 20 nucleotides of vector-encoded sequence. Samples were dissolved in 30 μl 80% formamide, containing 40 mM PIPES, 400 mM NaCl, 1 mM EDTA and 200,000 c.p.m. of the gel-purified transcript, denatured at 100 °C for 1 min and incubated at 45 °C for 20 h. RNase digestion was performed in 300 μl buffer containing 40 μg ml^{-1} RNase A (Sigma) and 2 μg ml^{-1} RNase T1 (Calbiochem) for 45 min at 37 °C. After digestion with proteinase K, samples were electrophoresed on denaturing 5% polyacrylamide gels.

controls but the difference was not statistically significant. Determination of prorenin showed it to be raised in the plasma of transgenic animals (Fig. 4*a*), but the functional significance of this finding is unclear. Adrenal glands of the transgenic animals contained significantly increased renin concentrations (Fig. 4*f*). No evidence was found for the storage of renin in this tissue, so the large difference between renin mRNA levels and enzyme activity may reflect a constitutive secretion of *Ren-2*-derived renin from the adrenal glands. By contrast, kidney tissue from transgenic animals contained only 20–25% of the renin activity of the controls, which is consistent with immunocytochemical and ultrastructural data showing a reduction in renin storage granules in the juxtaglomerular apparatus (S. Bachmann *et al.*, manuscript in preparation) and suggests that renin expression is subject to translational or post-translational control. Preliminary studies on isolated kidney show that renin secretion is reduced and that there are no other abnormalities of renal function (K. Munter, personal communication).

Although we have defined a genetic basis for this transgenic hypertensive rat model, the mechanism responsible for elevating blood pressure remains to be established. The hypertension is clearly not due to overexpression of renin in the kidney, and the suppression of active renin in the kidney and in the plasma is probably a result of an already elevated blood pressure in young animals, pressure-mediated renin suppression being a well known phenomenon. The increased plasma prorenin probably originates, at least in part, from the adrenal gland, but the ovary, vascular tissue and other sources of prorenin should also be considered. Any role of prorenin in hypertension still awaits investigation, but in this respect it is interesting that prorenin is raised and still persists after nephrectomy in hypertensive patients, confirming that its origin is extra-renal. At this stage, the most likely explanation for the high blood pressure in TGRmRen2 rats is a stimulated renin–angiotensin system in the adrenal gland, with the consequent overproduction of steroid hormones. This is in keeping with our preliminary data on

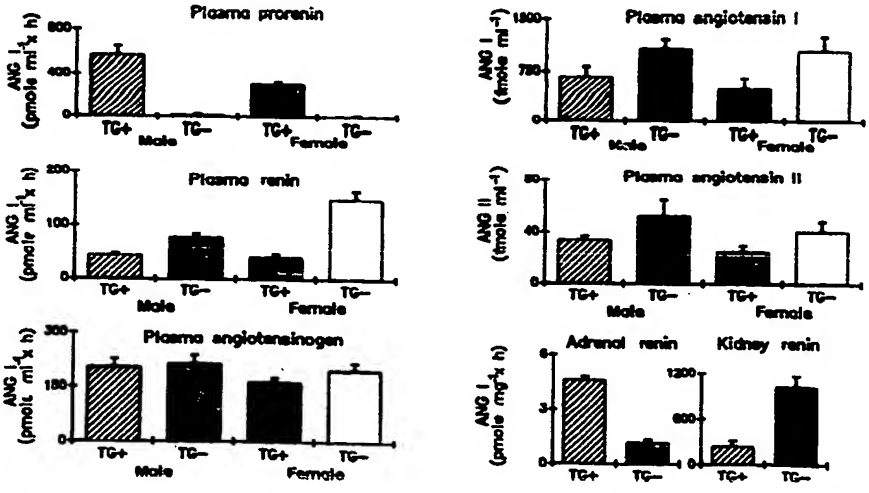

FIG. 4 Determination of plasma and tissue renin-angiotensin system components. Values represent the mean and standard error of 7 animals for each determination, with the exception of the kidney and adrenal gland renin values (3 animals). Statistical analysis by ANOVA showed the following significance values: prorenin, $P < 0.05$ between the transgenic animals and the corresponding controls; renin, $P < 0.005$ between the transgenic animals and the corresponding controls; angiotensin I, $P < 0.05$ between the transgenic animals and the corresponding controls; tissue renin, $P < 0.01$ for the adrenal gland and $P < 0.005$ for the kidney.

METHODS. Concentrations of angiotensinogen, angiotensin I, angiotensin II and renin were determined as described[24–25]. Prorenin levels were calculated by subtraction of renin activity from total plasma renin activity determined after trypsin activation[26].

elevated urinary aldosterone excretion in male TGRmRen2 rats (15.4 ± 2.26 ng per 24 h) compared with controls (8.97 ± 1.06 ng per 24 h). These animals will enable us to study normal or low plasma renin hypertension and have shown us that renin can participate in the genesis of hypertension in a more subtle way than previously supposed. The construction of transgenic rats will therefore provide new opportunities for research into cardiovascular mechanisms. □

Received 14 November 1989; accepted 9 February 1990.

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ACKNOWLEDGEMENTS. We thank Dr K. W. Gross for pCOS-1 and pSLM, Dr D. Armstrong for advice and communication of unpublished results, Frank Zimmerman and Gese Wernicke for technical assistance, Dr L. J. Mullins for critically reading the manuscript, and Drs U. Ganten, Yi Zhao and M. Lee for their contributions. This work was supported in part by the Deutsche Forschungsgemeinschaft, Squibb/von Heyden Co., Munich and The Commission of the European Communities, Concerted Action Program TRANSGENEUR.

Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses

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IN mammalian muscle, the subunit composition of the nicotinic acetylcholine receptor (AChR) and the distribution of AChRs along the fibre are developmentally regulated. In fetal muscle, AChRs are distributed over the entire fibre length whereas in adult fibres they are concentrated at the end-plate[1]. We have used *in situ* hybridization techniques to measure the development of the synaptic localization of the messenger RNAs (mRNAs) encoding the α-subunit and the ε-subunit of the rat muscle AChR. The α-subunit is present in both fetal and adult muscle, whereas the ε-subunit appears postnatally and specifies the mature AChR subtype[2–4]. The synaptic localization of α-subunit mRNA in adult fibres may arise from the selective down-regulation of constitutively expressed mRNA from extrasynaptic fibre segments. In contrast, ε-subunit mRNA appears locally at the site of neuromuscular contact and its accumulation at the end-plate is not dependent on the continued presence of the nerve terminal very early during synapse formation. This suggests that ε-subunit mRNA expression is induced locally via a signal which is restricted to the end-plate region and is dependent on the presence of the nerve only during a short period of early neuromuscular contact. Evidently, several mechanisms operate to confine AChR mRNAs to the adult end-plate region, and the levels of α-subunit and ε-subunit mRNAs depend on these mechanisms to differing degrees.

Hybridization of longitudinal sections of adult rat soleus muscle with ε- and α-subunit-specific antisense complementary RNA (cRNA) probes revealed strong hybridization signals at sites that had been previously identified as end-plates by staining for acetylcholinesterase (AChE). Figure 1*a* shows the end-plate region of a muscle stained for AChE. Subsequent hybridization with the ε-subunit-specific antisense probe showed a strong signal at the site where the AChE stain had been (Fig. 1*b*). After a brief exposure, groups of grains could be resolved above individual synaptic nuclei (Fig. 1*c*); no hybridization was observed outside end-plate regions. When sections were incubated with ε-subunit-specific sense probes, no hybridization could be detected (data not shown). These observations suggest that autoradiographic grain clusters reflect locally increased ε-subunit mRNA levels below the end-plate membranes. Similar results were obtained after hybridization with α-subunit-specific antisense (Fig. 1*d*, *e*) and sense probes and confirm the synaptic localization of α-subunit mRNA in rat muscle, as observed previously using northern blot analysis[5]. In some fibres, a small signal was observed above nuclei in the perijunctional region of the muscle fibres (Fig. 1*e*).

Previous northern blot analysis of AChR-specific mRNAs in neonatal rat muscle indicated that ε-subunit mRNA is barely detectable at birth but that levels increase rapidly during the first 2 weeks of postnatal development[4]. To determine whether this increase in ε-subunit mRNA is restricted to the end-plate region and therefore would be induced focally by the nerve, or whether the increase is more general, involving the entire fibre, we hybridized triceps muscle from rats of different postnatal ages with an ε-subunit mRNA-specific cRNA probe. Figure 2*a* shows the localization of AChE and autoradiographs of longitudinally sectioned muscle (*b–d*). At postnatal day 1, no hybridization signal could be detected either synaptically or extrasynaptically (Fig. 2*b*). In dark-field microscopy, some of the synaptic sites revealed a weak accumulation of grains (data not shown). However, on postnatal days 5, 9 (data not shown) and 12, an increasingly stronger signal was seen (Fig. 2*c*) that always coincided with the AChE-stained synaptic sites. Thus, the postnatal appearance of ε-subunit mRNA is restricted to the end-plate region from the earliest stages of synapse development and therefore must be induced by the nerve–muscle contact. As in adult muscle, hybridization signals in postnatal day-12 muscles were clearly associated with individual nuclei, as shown in Fig. 3. However, given the high density of nuclei from various cell types, unequivocal attribution to subneural nuclei was not always possible.

In contrast, total α-subunit mRNA remained at a plateau level during the first 12 postnatal days[4]. During this period, the α-subunit mRNA was detected throughout the fibre, in both the synaptic and extrasynaptic fibre segments (Fig. 2*f*, *g*). Although there were more grains at the synaptic sites, they were more widely distributed than those obtained upon hybridization with the ε-subunit mRNA specific probe. Moreover, the hybridization signal was also observed outside the myofibre bundles above unfused cells.

The level of total muscle ε-subunit mRNA increases almost normally in neonatal muscle denervated shortly after birth[4], indicating that only the brief, prenatal nerve–muscle contact is necessary to induce ε-subunit mRNA synthesis. We have investigated whether the ε-subunit mRNA still appears focally at the

Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3130–3134, May 1988
Immunology

Transgenic rabbits with lymphocytic leukemia induced by the c-*myc* oncogene fused with the immunoglobulin heavy chain enhancer

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Communicated by Leonard A. Herzenberg, December 21, 1987

ABSTRACT **Transgenic rabbits with the rabbit c-*myc* oncogene fused with the rabbit immunoglobulin heavy chain enhancer region (E_μ) DNA were developed by microinjecting pronuclei of single cell zygotes with the gene construct and implanting the microinjected eggs into pseudopregnant females. At age 17–20 days, 3 of 21 offspring born to seven females were found to have peripheral blood leukocyte counts of >100,000 per mm^3. Histology analyses showed extensive lymphocytic infiltration in the liver and kidney, indicating that these rabbits had a malignant lymphocytic leukemia. Genomic DNA analyses of thymus and peripheral blood lymphocytes revealed that the leukemic rabbits were transgenic and that both immunoglobulin heavy and κ light chain genes were rearranged in the leukemic cells; thus, the leukemic cells are of B-cell lineage. One to four heavy and light chain gene rearrangements were observed, suggesting that the B-cell tumors were oligoclonal. Stable tissue culture cell lines from the bone marrow and peripheral blood of one of the transgenic rabbits have been developed. The development of B-cell leukemias in rabbits with the E_μ-*myc* transgene contrasts with the development of B-cell lymphomas in mice carrying a similar transgene. The lymphomas in mice develop in adults and are monoclonal in origin. The leukemias in rabbits develop in juveniles, less than 3 weeks after birth, and appear oligoclonal in origin. The leukemias seem to develop in rabbit at a specific stage of development, and we suggest that a normal developmental signal may be involved in the oncogenesis.**

Segments of DNA that enhance or regulate gene transcription in a tissue-specific manner have been identified in association with several genes. These include those encoding immunoglobulin heavy and light chains (1–4), elastase I (5), α-fetoprotein (6), myosin light chain (7), β-globin (8, 9), insulin (10), and αA-crystallin (11). Transgenic mice that have received these tissue-specific regulatory elements fused with an oncogene have developed tumors of the tissue from which the DNA element was derived. For example, transgenic mice created with the simian virus 40 (SV40) early region DNA fused with a pancreatic enhancer segment developed pancreatic tumors (12). Similarly, transgenic mice receiving the SV40 oncogene fused with an insulin gene developed beta-cell tumors (10), transgenic mice receiving the SV40 oncogene fused with an αA-crystallin regulatory sequence developed lens tumors (13), and transgenic mice receiving the c-*myc* oncogene fused with the tissue-specific mammary tumor virus promoter and enhancer DNA segment developed breast adenocarcinomas (14).

The deregulated expression of the c-*myc* oncogene has been implicated in the development of lymphocytic tumors. Most murine plasmacytomas and human Burkitt lymphomas have c-*myc* translocations juxtaposing this cellular oncogene with immunoglobulin enhancer gene segments resulting in constitutive expression of the c-*myc* oncogene (15). Deregulated expression of the c-*myc* oncogene also results in the appearance of a variety of other tumors. One strain of transgenic mice harboring the mammary tumor virus promoter and enhancer gene segments fused with the c-*myc* gene develops testicular, mast cell, pre-B cell, B-cell, and T-cell tumors (16). Of particular interest are the experiments of Adams *et al.* (17) that demonstrate that transgenic mice harboring the c-*myc* gene fused with the immunoglobulin heavy chain enhancer region develop B-cell lymphomas. These observations raise the probability that development of c-*myc* transgenic animals of other species may increase our understanding of c-*myc* oncogenesis and possibly provide a source of B-cell tumors and B-lymphocyte cell lines. In the present study, we cloned the rabbit c-*myc* gene, localized the rabbit heavy chain enhancer (E_μ) region, and generated transgenic rabbits with a gene construct juxtaposing these two genes. We report here the development of lymphocytic leukemia in three transgenic rabbits at 17–20 days of age and the establishment of rabbit B-lymphoid cell lines.

MATERIALS AND METHODS

Rabbit c-*myc* Gene. The rabbit c-*myc* gene was cloned from the rabbit recombinant phage library X314-6 as described (18), with a 5.5-kilobase (kb) *Eco*RI fragment containing the v-*myc* gene used as probe (19). Positive clones were plaque purified and one, clone 14, was restriction mapped. The 5′ → 3′ orientation of the clone was determined by using appropriate v-*myc* probes and a synthetic DNA oligomer encoding 33 base pairs (bp) from a relatively conserved DNA sequence around the human and mouse c-*myc* TATAAT box (20).

Rabbit E_μ DNA Segment. The rabbit immunoglobulin E_μ DNA segment was identified by Southern analyses of rabbit DNA sequences and localized between the heavy chain joining region (J_H) and μ heavy chain constant (C_μ) region gene segments by using an oligonucleotide probe encoding the "core" enhancer DNA sequences derived from the mouse heavy chain enhancer region (2). A 0.6-kb *Bam*HI/*Eco*RI fragment within this intronic region of the rabbit heavy chain locus was examined further for enhancer activity by using a cDNA indicator gene, the tissue plasminogen activator gene, with the S107 immunoglobulin heavy chain variable region promoter. This gene construct is expressed poorly in transient expression experiments in B-lymphoid cells unless an enhancer DNA element is juxtaposed to the gene. Tissue plasminogen activator activity was measured by a technique to be published elsewhere.

Transgenic Rabbits. Adult female rabbits were obtained from Scientific Small Animals (Chicago). Rabbit zygote donors were injected subcutaneously with 50 international units of pregnant mare gonadotropin (Sigma) on day −4; immediately after mating, they were injected intravenously

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Abbreviations: PBL, peripheral blood leukocyte(s); E_μ, rabbit heavy chain enhancer; J, joining; C, constant.

with 150 international units of chorionic gonadotropin (Sigma). Single-cell zygotes were flushed from rabbit donor oviducts 19 hr later (day 1). The pronuclei were injected (21) with the 7.2-kb *Hin*dIII DNA fragment (1 μg/ml) containing the rabbit c-*myc* and E_μ DNA segments that had been cloned previously into pUC13; no vector sequences were present in the microinjected DNA. The injected zygotes were implanted through the fimbrial end of the oviduct of a recipient rabbit made pseudopregnant 2 days earlier by intravenous injection of 150 international units of chorionic gonadotropin or by mating with a sterile male.

Immunofluorescence and Histology Analyses. Membrane immunofluorescence staining was performed with hybridoma 2C4 anti-rabbit class II antibody and with goat anti-rabbit light chain antibody, as described (22). Cells were examined by using fluorescence microscopy and flow cytometry. Paraffin-embedded tissue sections of normal and leukemic rabbits were stained with hematoxylin/eosin.

Genomic DNA Analyses. Genomic DNA (5 μg), prepared by the method of Blin and Stafford (23), was analyzed by using methods described by Southern (24). DNA ^{32}P-labeled probes were prepared as described (18). The 2.7-kb *Sac* I/*Bgl* II rabbit κ light chain (C_κ) genomic probe was provided by E. Max (National Institutes of Health). Hybridization analyses with ^{32}P-labeled oligomeric probes were performed in 0.9 M NaCl/0.09 M sodium citrate at 12°C for 18 hr.

Tissue Culture. Single-cell suspensions for tissue culture were prepared from spleen, mesenteric lymph nodes, and bone marrow. Peripheral blood leukocytes (PBL) were prepared with Seprapette-MN tubes (Sepratech, Oklahoma City, OK). All cells were grown in RPMI 1640 medium with 15% fetal calf serum, 5% normal rabbit serum, 0.5 mM 2-mercaptoethanol, and 1 mM sodium pyruvate. Additional supplements included supernate of interleukin 3-producing WEHI-3B cells (25), supernate of B-cell-stimulatory factor 1-producing D10.G4.1 cells (26), and supernate of concanavalin A (Con A)-stimulated rabbit spleen cells.

RESULTS

Identification of the Rabbit c-*myc* Gene and the Rabbit E_μ. A 6.6-kb *Bam*HI fragment was found to hybridize with v-*myc* and was further analyzed by DNA hybridization (Fig. 1*A*) with 3′ and 5′ probes of v-*myc*. The c-*myc* exon 1 was localized by hybridization with the 33-bp oligomer encoding sequence neighboring the TATAAT box of the human *MYC* gene.

A 0.6-kb *Bam*HI/*Eco*RI fragment ≈1 kb downstream of the J_H region cross-hybridizes with the mouse core enhancer

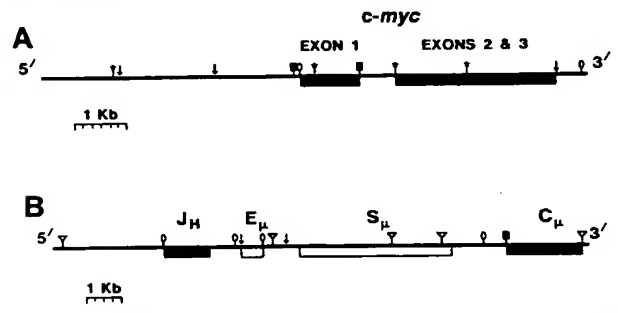

FIG. 1. Restriction maps of rabbit c-*myc* recombinant phage clone (*A*) and rabbit J_H-C_μ chromosomal region (*B*). The solid boxes in *A* represent regions that hybridized with the c-*myc* exon 1 oligomer probe (5′ solid box) and the v-*myc* probe (3′ solid box). The regions encoding J_H segments and C_μ are designated in *B* by solid boxes. The E_μ and the switch region (S_μ) are designated by open boxes. The enzyme symbols are as follows: ♀, *Bam*HI; ↓, *Eco*RI; ↑, *Sac* II; ▽, *Hin*dIII; and ♦, *Xho* I.

oligonucleotide probe (Fig. 1*B*). The murine heavy chain enhancer region also is found ≈1 kb downstream of the murine J_H region. The 0.6-kb *Bam*HI/*Eco*RI fragment showed functional enhancer activity when tested in conjunction with the tissue plasminogen activator indicator gene (data not shown). This 0.6-kb *Bam*HI/*Eco*RI fragment was cloned 3′ to the 6.6-kb *Bam*HI c-*myc* gene and the resulting construct was used for the microinjections.

Development of Transgenic Rabbits and of Rabbit Lymphocytic Leukemias. Two hundred and forty-six zygotes injected with the transgene construct were implanted together with 43 uninjected zygotes into a total of 15 pseudopregnant females. Seven of the females became pregnant and delivered a total of 21 live offspring and 10 dead fetuses. Three of the 21 offspring were found to be leukemic at 17–20 days of age, having PBL counts of 500,000 cells per mm^3 (rabbit K74-4), 600,000 cells per mm^3 (rabbit K75-4), and 100,000 cells per mm^3 (rabbit K47-4). These values contrast with PBL values of ≈5000 cells per mm^3 in normal rabbits. The three animals were sacrificed and Southern analyses of *Bam*HI-digested liver and PBL DNA revealed the presence of multiple copies of the 6.6-kb c-*myc* transgene (Fig. 2, data for K47-4 not shown). The endogenous c-*myc* gene is found on a 25-kb fragment in the genomic DNA of each of the animals tested. Seventeen other rabbits lived beyond the age of 2 months, at which time DNA was isolated from PBL and examined by Southern analyses for the presence of the transgene. Each of these 17 rabbits had the endogenous c-*myc* gene but no transgene (data not shown).

Pathology and Histology of Leukemic Transgenic Rabbits. The three transgenic rabbits (K74-4, K75-4, and K47-4) presented with palpable splenomegaly and high numbers of PBL on day 17 (K74-4), day 18 (K75-4), and day 20 (K47-4). Necropsy revealed that the spleen, liver, and kidneys were enlarged. Examination of blood smears (Fig. 3) confirmed the increased PBL count and revealed a population of large atypical lymphocytes (lymphoblasts). Most of these neoplastic cells contained vacuoles and split- or cloverleaf nuclei. The histology of the three transgenic rabbits was identical and revealed that the bone marrow, kidney, liver, and spleen were infiltrated by the neoplastic lymphoblasts (Fig. 4).

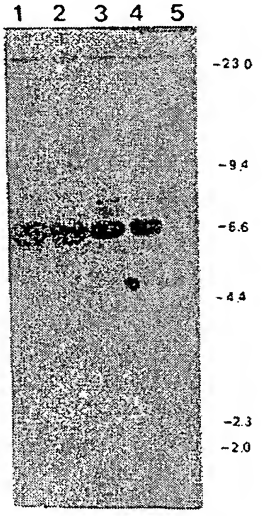

FIG. 2. Southern blot analysis of thymus and PBL DNA restricted with *Bam*HI from rabbits K74-4 and K75-4. The blot was hybridized with the 6.6-kb *Bam*HI rabbit germ-line c-*myc* probe. Lane 1, K74-4 PBL; lane 2, K74-4 liver; lane 3, K75-4 PBL; lane 4, K75-4 liver; lane 5, normal rabbit sperm DNA. The sizes (kb) of *Hin*dIII-digested λ phage are shown as size standards.

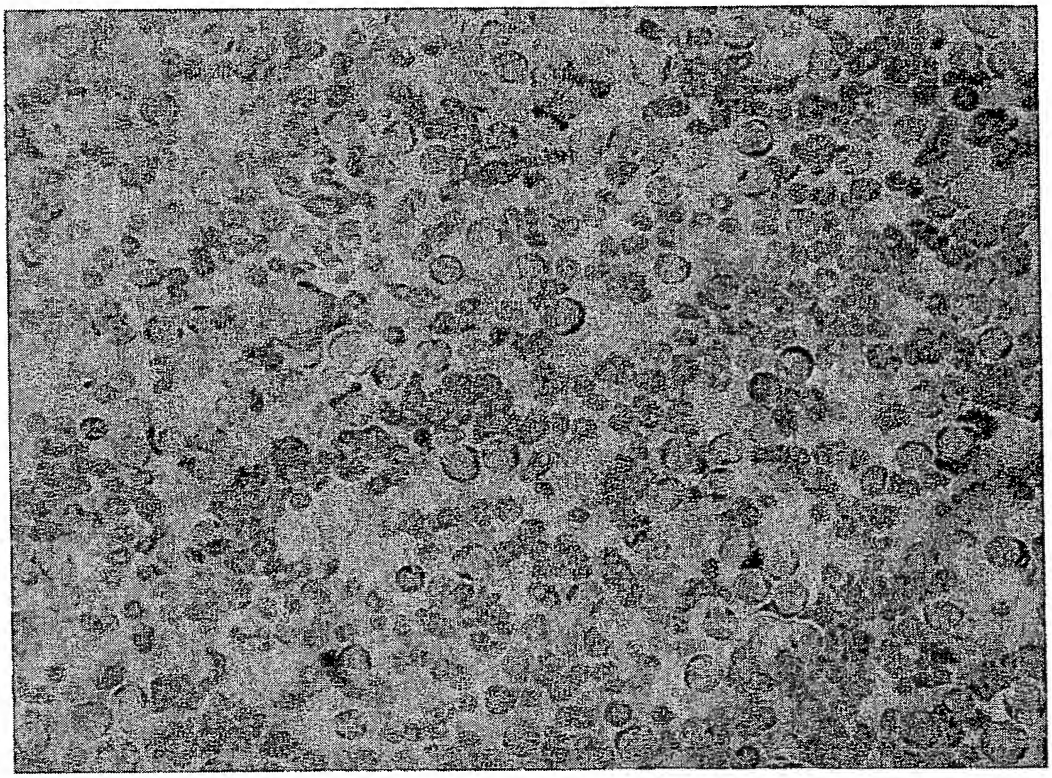

FIG. 3. Blood smear of rabbit K74-4 stained with hematoxylin/eosin.

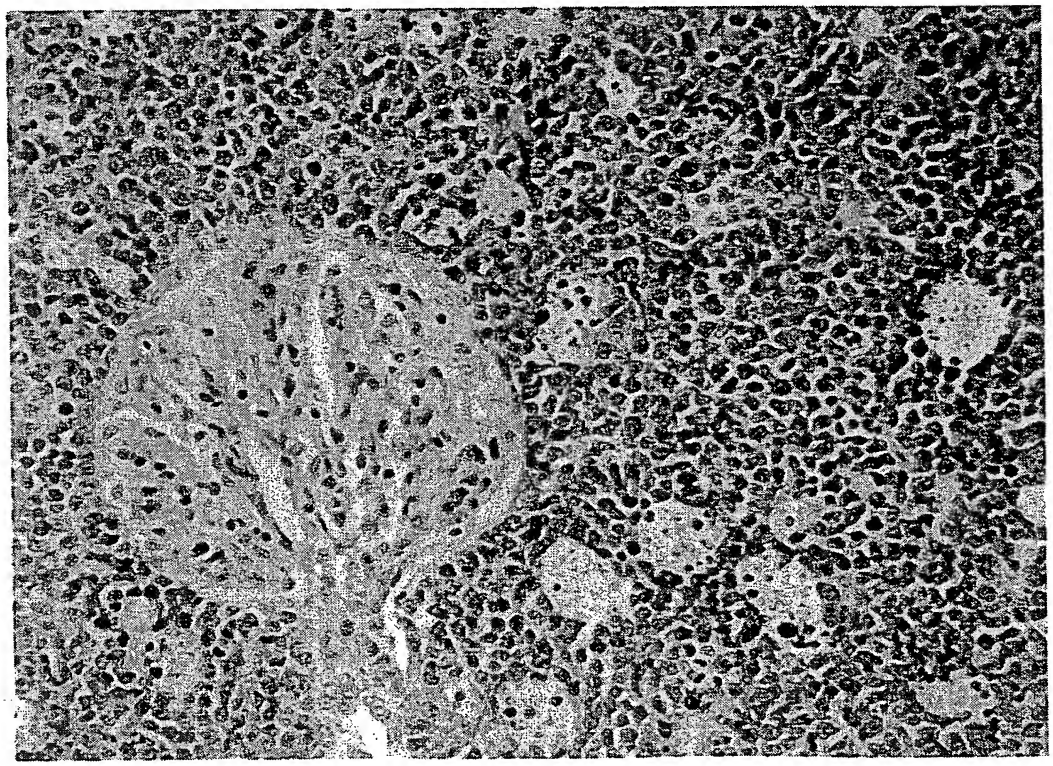

FIG. 4. Section of kidney of rabbit K75-4 stained with hematoxylin/eosin.

Rabbit Tissue Culture Cell Lines. Cells from various tissues of the three transgenic rabbits were placed into culture to establish cell lines. After 2–6 weeks in culture, the lymphoid cells of rabbits K74-4 and K75-5 were no longer viable and only fibroblast-like cells remained. Viable lymphoid cells began to emerge from the bone marrow cells of rabbit K47-4 after 10 days in culture and from PBL after 3 weeks in culture. The bone marrow cells grew from all cultures except those that contained Con A supernate. The PBL grew under all culture conditions; however, cells from the cultures containing Con A supernate grew considerably faster than those that did not contain Con A supernate. Cells from the bone marrow and PBL have been adapted to grow in RPMI 1640 medium containing 10% fetal calf serum and 5% normal rabbit serum without additional supplements. These cells have continued to divide in culture for >1 year. The doubling time of bone marrow-derived and PBL-derived cells is about 24 hr.

Immunofluorescent and Molecular Genetic Analyses of the Rabbit Leukemic Cells. Membrane immunofluorescence staining of PBL of rabbits K74-4 and K75-4 revealed that all cells reacted with the anti-rabbit class II hybridoma antibody 2C4. These cells reacted weakly, if at all, with anti-rabbit immunoglobulin antibody. Similarly, the bone marrow-derived and PBL-derived cell lines of rabbit K47-4 reacted with the anti-class II antibody and showed little or no reactivity with the anti-immunoglobulin antisera. These cell lines do not appear to be mature B-lymphocytes.

Southern analyses of genomic DNA from liver, thymus, and PBL of the leukemic rabbits were done by using rabbit J_H and C_κ probes. Hybridization of *Hin*dIII-restricted DNA with the J_H probe revealed that J_H rearrangements had occurred in the PBL of all leukemic rabbits. PBL DNA of rabbit K74-4 had two non-germ-line J_H hybridizing fragments of 4.1 kb and 3.2 kb; the weakly hybridizing 4.1-kb band in thymocyte DNA probably reflects contamination of the thymus with infiltrating leukemic cells. PBL DNA of rabbit K75-4 had four J_H hybridizing bands—9.7 kb, 4.7 kb, 4.2 kb, and 4.1 kb—not found in thymocyte (presumed germ line) DNA (Fig. 5*A*). PBL DNA of rabbit K47-4 had one non-germ-line J_H hybridizing fragment, 4.1 kb (data not shown). In all cases, the appearance of additional J_H hybridizing fragments in PBL DNA was accompanied by loss of hybridization intensity to one or both germ-line J_H fragments. The J_H gene rearrangements strongly suggest that the leukemic cells in all three rabbits are of the B-lymphoid lineage.

Similar studies with the C_κ probe indicated that the $C_\kappa 1$ gene was rearranged in all three leukemic rabbits (Fig. 5*B*). Southern analyses of *Hin*dIII-restricted DNA using a C_κ probe revealed the intensity of hybridization of the functional $C_\kappa 1$ gene (6.5-kb *Hin*dIII fragment) was decreased in the PBL DNA, relative to thymocyte and normal liver DNA. PBL DNA from the transgenic rabbits had four or five new C_κ hybridizing fragments, indicating that several different C_κ gene rearrangements had occurred and that the leukemias were oligoclonal. As a control, the intensity of hybridization with the infrequently expressed $C_\kappa 2$ gene found on a 10-kb *Hin*dIII fragment (27) was observed to be similar in leukemic thymus, PBL, and liver DNA.

Southern analysis of DNA from two different lines of cultured PBL cells from rabbit K47-4 revealed one germ-line J_H hybridizing fragment and a 4.8-kb *Hin*dIII rearranged J_H fragment; hybridization with the C_κ probe revealed one cell line (no. 2) with germ-line $C_\kappa 2$ and a rearranged C_κ (presumably $C_\kappa 1$) 18-kb *Hin*dIII fragment. The other cell line (no. 1) had germ-line $C_\kappa 2$ and two rearranged $C_\kappa 1$ fragments of 18 kb and 9.8 kb (Fig. 6).

DISCUSSION

B-lymphocytic leukemias have been developed in transgenic rabbits harboring the E_μ-*myc* transgene. Studies with mice carrying a similar transgene have shown that such mice develop B lymphomas (17). Two major differences between the oncogenic outcomes in rabbit and in mouse are noted. The rabbit leukemias consistently occurred early in development, 17–20 days of age, and the leukemic cells appear oligoclonal in origin. In contrast, the murine lymphomas occurred late in development, months of age, and are monoclonal. Langdon *et al.* (28) have shown that polyclonal proliferation of pre-B cells occurs in E_μ-*myc* mice. Presumably, c-*myc* expression, driven by E_μ of the transgene, occurs polyclonally, early in B-cell development. Langdon *et al.* (28) suggest that oncogenesis that occurs in the adult transgenic mice requires another event, a "genetic accident, such as activation of a second oncogene." Since this "second signal" is apparently a random event, it results in a monoclonal

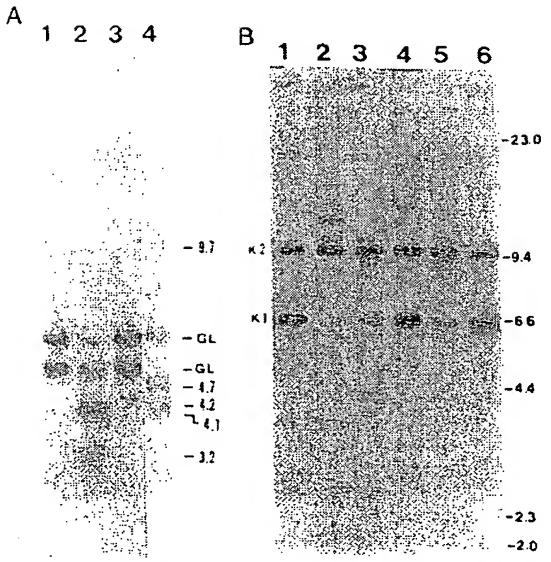

FIG. 5. Southern blot analysis of transgenic rabbit DNA restricted with *Hin*dIII. (*A*) The blot was hybridized with a rabbit J_H probe. Lane 1, K74-4 thymus; lane 2, K74-4 PBL; lane 3, K75-4 thymus; lane 4, K75-4 PBL. The intense bands of thymus DNA are presumably in germ-line configuration. The sizes (kb) of germ-line (GL)-sized hybridizing fragments are indicated. (*B*) The blot was hybridized with rabbit C_κ probe. Lane 1, K74-4 thymus; lane 2, K74-4 PBL; lane 3, K75-4 liver; lane 4, K75-4 thymus; lane 5, K75-4 PBL; lane 6, normal rabbit liver. The sizes (kb) of *Hin*dIII-digested λ phage are shown as size standards. Germ-line K1 and K2 fragments are indicated.

FIG. 6. Southern blot analysis of DNA from two cultured cell lines from rabbit K47-4. (*A*) Probe J_H. (*B*) Probe C_κ. Lanes 1 and 2, cell lines 1 and 2, respectively. GL, germ line. Sizes (kb) refer to non-germ-line-sized hybridizing fragments. K2, germ-line K2.

malignancy. In E_μ-*myc* rabbits, the consistent early appearance of malignancies and their oligoclonal nature suggest either that an early developmental signal is involved in B-cell oncogenesis or that the E_μ-*myc* transgene alone is sufficient. We propose that a normal developmental or growth factor functions in a polyclonal manner as a second signal for promotion of oncogenesis, resulting in malignancies that are oligoclonal in nature. Thus, it appears that different second signals are involved in oncogenesis in E_μ-*myc* rabbits and mice. It is likely that these different second signals are responsible for the phenotypic differences in the murine and rabbit malignancies, lymphomas and leukemias, respectively.

Although the rabbit leukemias appear to be of the B-cell lineage, as they had rearranged immunoglobulin heavy and κ light chain genes, the precise stage of differentiation of these cells is difficult to assign. The cells express major histocompatibility complex class II molecules but have little or no surface immunoglobulin. Preliminary results indicate that the cells adapted to tissue culture secrete low levels of κ light chain but little or no heavy chain. These cells may be similar to chronic lymphocytic leukemia cells that also have little or no surface immunoglobulin and secrete low levels of light chains (29).

Southern blots of restricted DNA from the transgenic rabbits showed multiple rearrangements of the J_H gene segments, indicating that the leukemias were oligoclonal. We do not know, however, whether these J_H rearrangements represent productive or nonproductive rearrangements. The two cell lines in culture from rabbit K47-4 had different C_κ rearrangements, indicating that the cell lines were derived from two different clones; however, it is possible that these lines are progeny of the same clone since one of the rearranged C_κ genes of each clone appears to be identical. The rearranged J_H genes in these cultured cell lines were different from the rearrangement seen in the DNA of PBL of that rabbit. This suggests that there were J_H rearrangements in PBL DNA that were not detected by Southern analysis and that cells with these rearrangements preferentially grew out in tissue culture. We cannot, however, rule out the possibility that these cultured cells are derivatives of a single progeniter clone whose J_H rearrangement was observed in Southern blots of PBL DNA at necropsy and that J_H rearrangements took place *in vitro*.

Lymphocytic tumors in laboratory rabbits have rarely been observed. Although several lymphosarcomas were reported in a particular colony of rabbits (30), only two rabbit lymphocytic leukemias have been reported (31, 32). The low incidence of rabbit lymphoid tumors explains the paucity of rabbit T- and B-cell lines. In fact, the only previously reported rabbit B-lymphocyte line in culture was one developed by Collins *et al.* (33). The E_μ-*myc* transgenic rabbits should provide a source of B-cell lines and these cells will provide the opportunity to study B-cell differentiation and immunoglobulin gene rearrangements. Most importantly, these malignant cells can potentially be used to develop a fusion partner for the generation of rabbit somatic cell hybrids.

We are indebted to Dr. Susan Ross for teaching us the microinjection technique and to John Zaryczny for his expert animal husbandry skills. We thank Alan Golden for surgical help and Dr. Marilyn Brown for her veterinary help and advice. Furthermore, we are grateful to Dr. Robert Wall (Department of Agriculture Agricultural Research Service, Beltsville, MD) for helpful discussions regarding the development of transgenic rabbits. This research was supported in part by Public Health Service Grant AI16611.

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